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**MICROBIAL PROCESSES CONTRIBUTING TO N<sub>2</sub>O  
PRODUCTION IN TWO SANDY SCOTTISH SOILS**

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Thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Science  
and Engineering, Institute of Ecology and Resource Management

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1997





## **DECLARATION**

I, Simona Castaldi, hereby declare that this thesis was composed by myself, and that the work described was carried out by myself, except for the instance detailed in the acknowledgements. The work presented here has not been submitted in any previous application for a degree.

Simona Castaldi

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## ABSTRACT

The objective of the present work was to investigate the potential contribution of different microbial processes to  $\text{N}_2\text{O}$  fluxes and the influence of the main environmental factors on these processes in two light textured Scottish soils. Two imperfectly drained brown forest soils of the Peffer Series, derived from fine beach sand, were studied. One was a sandy loam, sampled from a deciduous woodland and the other was a loamy sand, sampled from a nearby winter wheat field, at Gullane, East Lothian, Scotland. Both soils had slightly alkaline pHs.

Field and laboratory studies demonstrated that more than one process was responsible for  $\text{N}_2\text{O}$  emissions. Field results showed two different patterns of  $\text{N}_2\text{O}$  emissions. Measurements with static manual chambers showed very low fluxes ( $1.4 - 1.5 \text{ g N}_2\text{O-N ha}^{-1} \text{ d}^{-1}$ ) throughout the year, though with some seasonal variation. These fluxes were not correlated with any environmental parameter measured. However, measurements with automatic chambers occasionally showed higher  $\text{N}_2\text{O}$  fluxes (up to  $44 \text{ g N}_2\text{O-N ha}^{-1} \text{ d}^{-1}$ ). The latter were favoured by the presence of fresh organic matter and high concentrations of  $\text{NH}_4^+\text{-N}$  (rather than  $\text{NO}_3^-\text{-N}$ ). This was shown to be true both in a fertilization experiment in the woodland and in an incubation experiment with woodland soil cores. The nature of the processes responsible for the  $\text{N}_2\text{O}$  field emissions, e.g. denitrification, autotrophic and heterotrophic nitrification, are discussed and it is concluded that nitrification (either autotrophic or heterotrophic) was mainly responsible for the low  $\text{N}_2\text{O}$  fluxes, whereas denitrification or heterotrophic nitrification-denitrification was the main source of the higher fluxes.

$\text{N}_2\text{O}$  emissions, determined in a laboratory experiment in which the soils were incubated with different sources of nitrogen, with or without glucose, and with 0, 1 and  $100 \text{ ml C}_2\text{H}_2 \text{ l}^{-1}$ , showed large differences in the rate of  $\text{N}_2\text{O}$  production both between the two soils and between the different N treatments. The arable soil showed very low  $\text{N}_2\text{O}$  emissions derived from reduced forms of N as compared with the  $\text{N}_2\text{O}$  which was produced when the soil was provided with  $\text{NO}_2^-$  or  $\text{NO}_3^-$  and a C source, suggesting a very active denitrifier population. In contrast, the woodland soil showed a very low denitrification activity and a much higher  $\text{N}_2\text{O}$  production derived from the oxidation of  $\text{NH}_4^+$  and reduction of  $\text{NO}_2^-$  by some processes probably mediated by autotrophic or heterotrophic nitrifiers. In both soils, the highest  $\text{N}_2\text{O}$  emissions were induced by  $\text{NO}_2^-$  addition. Those emissions were demonstrated to have a biological origin, as no significant  $\text{N}_2\text{O}$  emissions were measured when the soil was autoclaved.

Experiments carried out using  $^{15}\text{N}$  pool dilution and enrichment techniques and physiological block techniques for prokaryotic and eukaryotic microorganisms (streptomycin and cycloheximide, respectively) showed that a well established population of heterotrophic nitrifiers was present in the woodland soil. The balance between autotrophic and heterotrophic nitrification in the soil was influenced by the concentration of organic N. The heterotrophic activity increased from 18% to 56% of the total nitrification activity when the peptone concentration was increased from  $70$  to  $280 \mu\text{g N g}^{-1}$ .

Low concentrations ( $0\text{--}2.5\text{ mg g}^{-1}$ ) of both antibiotics had no apparent biocidal and disruptive effect on the microbial biomass, in the first 48 h incubation, indicating only a selective action of protein synthesis inhibition, whereas at high concentration ( $7.5\text{ mg g}^{-1}$ ) cycloheximide had a marked biocidal effect on the overall population of nitrifiers, blocking completely any nitrification activity.

Heterotrophic nitrification was completely blocked and autotrophic nitrification was reduced at  $2\text{ mg cycloheximide g}^{-1}$ , while streptomycin only slightly reduced both autotrophic and heterotrophic nitrification, even at  $3.5\text{ mg g}^{-1}$ . This suggested that fungi could have a dominant role in  $\text{NO}_3^-$  production from readily available organic-N in the woodland soil, even at slightly alkaline pH. The partial inhibition of autotrophic nitrification by low concentrations of cycloheximide indicate the possibility for another fungal pathway of  $\text{NO}_3^-$  production which might utilize an inorganic route. This possibility was also supported by the results with non-isotopic techniques, where the  $\text{N}_2\text{O}$  fluxes induced by peptone addition were completely inhibited by low concentrations of cycloheximide ( $1\text{--}2\text{ mg cycloheximide g}^{-1}$ ) but also by  $0.1\%$  ( $100\text{ Pa}$ ) acetylene, suggesting a possible role of ammonia monooxygenase in an organic-inorganic pathway of nitrification in fungal metabolism.

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AS	Ammonium sulphate
C	Control
CN	Calcium nitrate
Cx	Cycloheximide
ECD	Electron capture detector
FTIR	Fourier transform Infrared spectrometer
G	Glucose
GC	Gas chromatograph
P	Peptone
PVC	Polyvinylchloride
SN	Sodium nitrite
Str	Streptomycin sulphate
TCD	Termo-conductivity detector
U	Untreated
WFPS	Water filled pore space
WHC	Water holding capacity
WL	Water logged

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## **INTRODUCTION**

### **1.1 CLIMATE CHANGE, A MAJOR ENVIRONMENTAL ISSUE**

Climate change is at present one of the major issues in international environmental research. Since problems such as the greenhouse effect and climate warming were first brought to the attention of the scientific audience, many efforts have been made to give a critical evaluation of the problem and make reliable predictions about climate change. The issue is, however very controversial, because of the difficulty to prove cause and effects and the complexity of the mitigating measures (scientifically, politically and economically speaking) (Gates, 1993).

Nevertheless, there is now general agreement that human activities are increasing atmospheric concentrations of greenhouse gases (which tend to warm the atmosphere) and aerosols (which tend to cool the atmosphere). Taken together, these changes are predicted to lead to regional and global change of climate (temperature, precipitation, sea level, etc.). Prediction based on models indicate for the year 2100 a rise in mean surface temperature of 1 - 3.5 °C and an associated increase in the sea level of about 15 - 95 cm. Human health, terrestrial and aquatic ecosystems and socio-economic systems are all likely to experience adverse effects of climate change (IPCC, 1995. The Science of Climate Change).

## 1.2 GREENHOUSE GASES AND CLIMATE WARMING

The earth with its atmosphere acts as a greenhouse. Solar radiation of wavelengths between 0.3 and 4  $\mu\text{m}$  is absorbed by the atmosphere and by the earth's surface; for climate to be in equilibrium, this absorbed solar radiation must be balanced by outgoing thermal radiation (4 to 100  $\mu\text{m}$  wavelength). Radiatively-absorbing particles and molecules, present mainly in the first 10 - 15 km of the atmosphere, by partially trapping and remitting the outgoing thermal radiation (at approximately 8-14  $\mu\text{m}$ ) induce an increase of surface temperature of about 33°C (as compared with on absence of any atmosphere). This phenomenon is known as the *GREENHOUSE EFFECT*. Though clouds and water vapour are the main contributors to this process, other gases present at low or trace concentrations contribute significantly to the greenhouse effect. These so-called *trace gases* include carbon dioxide ( $\text{CO}_2$ ), methane ( $\text{CH}_4$ ), tropospheric ozone ( $\text{O}_3$ ), nitrous oxide ( $\text{N}_2\text{O}$ ) and some chlorofluorocarbons (CFC-11, CFC-12) (IPCC, 1990). While clouds and water vapour dynamics follow a natural climatic pattern (atmospheric hydrogeological cycle), human activities have significantly increased the atmospheric concentration of these trace gases during the last century (Table 1.1).

Increasing atmospheric concentrations of  $\text{CH}_4$ ,  $\text{N}_2\text{O}$ ,  $\text{O}_3$ , CFC-11 and CFC-12 are particularly detrimental, as these gases have a strong absorption in a spectral region from 8 to 12  $\mu\text{m}$  known as the "window", because of the relative atmospheric transparency to radiation over these wavelengths, which allows about 25% of the thermal emission of the Earth's surface to escape into space (Dickinson and Cicerone, 1986).

Though the concentration of  $\text{N}_2\text{O}$  is small as compared with that of  $\text{CO}_2$ , the global warming potential of each molecule of  $\text{N}_2\text{O}$  is about 200 times greater than each molecule of  $\text{CO}_2$  on either a mass or a mole basis (Gates, 1993). The actual concentration of nitrous oxide is about 311 ppbv (giving an atmospheric total of about 1500 Tg N) and is increasing at a rate of 0.25% per year. This concentration is higher

in the Northern Hemisphere than in the Southern one by about 1 ppbv. N<sub>2</sub>O lifetime is about 120 years, which has implications for achieving stable concentrations.

**Table 1.1** - Estimated pre-industrial and “1992” concentrations of trace gases and their annual increase (Modified from IPCC 1995, *The Science of Climate Change*).

Gas	Pre-industrial concentration (ppbv)	Concentration in 1992 (ppbv)	Radiative absorption potential	Life time (years)	Contribution to greenhouse warming	Annual increase (ppbv/yr)
CO <sub>2</sub>	278,000	356,000	1	variable	50	1,600
CH <sub>4</sub>	700	1714	32	12.2	19	8
N <sub>2</sub> O	275	311	200	120	4	0.8
CFC-11	0	0.268	>10000	50	15 (both CFCs)	+ 0.000
CFC-12	0	0.503	“	102		+ 0.007

If emissions were held constant at today’s level, N<sub>2</sub>O abundance would continue to grow for hundreds of years up to 400 ppbv. To stabilize N<sub>2</sub>O concentrations at the current level, anthropogenic sources would need to be reduced by more than 50% (IPCC, 1994).

### 1.3 GLOBAL SOURCES AND SINKS FOR N<sub>2</sub>O AND THE ROLE OF TERRESTRIAL ECOSYSTEMS

A great number of N<sub>2</sub>O sources have been identified (Table 1.2), however the uncertainty of the estimate source strength is still big.

Terrestrial ecosystems represent the main source of N<sub>2</sub>O, the natural ones accounting for about 6 Tg(N)/yr and the cultivated soils for 3.5 Tg(N)/yr (Table 1.2). Tropical soils (forests and savannahs) are the single most important source of N<sub>2</sub>O, emitting 4 Tg(N)/yr (range 2.7-5.7). Recently converted pastures have been shown to emit 3-10 times more N<sub>2</sub>O than tropical forest, while the opposite has been observed for old pastures (> 10 years) (Keller *et al.*, 1993). The magnitude of N<sub>2</sub>O emissions from intensively fertilized tropical agricultural soils has not been quantified.

**Table 1.2** - Estimated sources and sinks of N<sub>2</sub>O typical of the last decade (Tg(N)/yr). (From IPCC, 1994).

	Range	Likely
<b>Atmospheric increase</b>	3.1 - 4.7	3.9*
<b>Sinks</b>		
stratosphere	9 - 16	12.3
soils	?	
<b>Total Sinks</b>	9 - 16	12.3
<b>Implied total sources</b> (atmospheric increase + total sinks)	13 - 20	16.2
<hr/>		
Identified sources	Range	Likely
<b>Natural</b>		
oceans	1 - 5	3
<i>tropical soils</i>		
wet forests	2.2 - 3.7	3
dry savannahs	0.5 - 2.0	1
<i>temperate soils</i>		
forests	0.1 - 2.0	1
grasslands	0.5 - 2.0	1
<b>Total identified natural sources</b>	6 - 12	9
<b>Anthropogenic</b>		
cultivated soils	1.8 - 5.3	3.5
biomass burning	0.2 - 1.0	0.5
industrial sources	0.7 - 1.8	1.3
cattle and feed lots	0.2 - 0.5	0.4
<b>Total identified anthropogenic</b>	3.7 - 7.7	5.7
<b>TOTAL IDENTIFIED SOURCES</b>	<b>10 - 17</b>	<b>14.7</b>

\*The observed atmospheric increase implies that sources exceed sinks by 3.9 Tg(N)/yr.

A relatively smaller amount of N<sub>2</sub>O comes from grassland and from temperate forest soils, for which an average emission of 1 Tg(N)/yr has been calculated (Table 1.2). The deposition of N into natural ecosystems such as forest and moorland can contribute significantly to N<sub>2</sub>O release, especially in the Northern Hemisphere where forest soils are often not too far from highly industrialized areas or other sources of atmospheric N (such as ammonium from animal urine). Dramatic effects due to N deposition in forest soils have been observed in Germany (Schmidt *et al.*, 1988). The global deposition of N has been estimated as 40-116 Tg N yr<sup>-1</sup> as nitrate (Rosswal T., 1983) and 50-128 Tg N yr<sup>-1</sup> as NH<sub>4</sub><sup>+</sup>/NH<sub>3</sub> (Schlesinger and Hartley, 1992). When N deposition is accompanied by soil acidification the N<sub>2</sub>O fraction of the gaseous

products of denitrification is increased as reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$  is inhibited. Liming has resulted in much lower fluxes (Brumme and Beese, 1992). However, not always forests receiving high N inputs have been found to increase  $\text{N}_2\text{O}$  production (Castro *et al.*, 1993).

About 40% of the sources originate from anthropogenic activity. Among the smaller sources, a significant contribution comes from burning, which includes burning of forests during clearing, natural forest fires, seasonal savannah burning, shifting cultivation, agricultural residue and firewood burning. Biomass burning not only produces  $\text{N}_2\text{O}$  instantaneously; it also enhances, in the longer term,  $\text{N}_2\text{O}$  production from soil by altering the quality and availability of nutrients and organic matter (Anderson *et al.*, 1988; Levine, 1988).

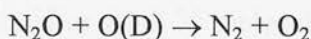
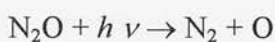
A bigger contribution comes from cultivated soils, where the main emissions of  $\text{N}_2\text{O}$  arise from fertilization with mineral N and animal manure, N derived from N-fixation (legumes and N-fixing microbes) and N from enhanced soil mineralization (Duxbury and Mosier, 1993). The indirect contribution of fertilizer N to  $\text{N}_2\text{O}$  emissions is also important, as an estimated additional 0.75% of N applications will be lost as  $\text{N}_2\text{O}$  resulting from leaching, runoff, nitrogen oxides ( $\text{NO}_x$ ) and  $\text{NH}_3$  volatilization (Minami and Ohsawa, 1990; Duxbury *et al.*, 1993; Mosier, 1993). Soil management and cropping systems and rainfall inputs have been found to affect  $\text{N}_2\text{O}$  emissions more than mineral N sources (Mosier, 1993). As  $\text{N}_2\text{O}$  fluxes from agricultural systems are highly variable in both time and space (Smith, 1990; Clayton *et al.*, 1994, McTaggart *et al.*, 1994) prediction of the  $\text{N}_2\text{O}$  emissions associated with a unit of added N fertilizer or fixed N by legume, is not yet reliable. However, an estimation of total emissions of  $\text{N}_2\text{O}$ , based on a recent review of published data, has been provided by Bouwman (1996) based on the following regression equation:

$$\text{Total annual direct field } \text{N}_2\text{O-N loss} = 1 + 0.0125 \times \text{N-application (kg N/ha)}$$



The direct and indirect N<sub>2</sub>O-N emissions from mineral and organic N applications are approximately  $2 \pm 1\%$  annually (Mosier *et al.*, in press). This estimate is expected to encompass more than 90% of field situations.

Atmospheric reactions represent the major N<sub>2</sub>O sink (9-16 Tg(N)/yr) (Table 1.2) In the stratosphere N<sub>2</sub>O is destroyed by the reaction with excited singlet oxygen atoms formed by the photolysis of ozone and is transformed into nitric oxide (NO), which participates in further reactions with ozone, contributing to the reduction of the ozone layer in the stratosphere (Crutzen, 1981). In the stratosphere N<sub>2</sub>O is also removed by the following reactions (Crutzen, 1983) :



A minor sink for N<sub>2</sub>O is represented by soil. Only very small rates of N<sub>2</sub>O uptake have been observed in some dry soils (Duxbury and Mosier, 1993) and in wet grass pastures (Ryden 1981). Anaerobic soils have a large potential to act as a sink for N<sub>2</sub>O (Erich *et al.*, 1990); however, this sink is likely to be small, because the essential condition, i.e. wet soil, creates a diffusion barrier to entry of N<sub>2</sub>O from atmosphere. To date, the magnitude of such a sink as not yet been evaluated. With the current knowledge, N<sub>2</sub>O sources are estimated to be almost 15% in excess over N<sub>2</sub>O sinks.

## 1.4 PROCESSES WHICH PRODUCE N<sub>2</sub>O

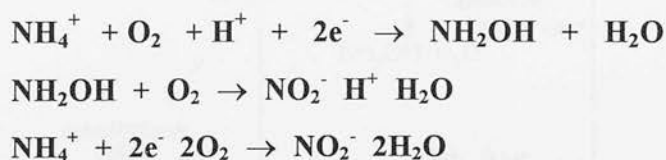
### 1.4.1. AUTOTROPHIC NITRIFICATION

The nitrification process can be considered as the biological oxidation of reduced forms of nitrogen to more oxidised ones. The most diffused pathway of nitrification is the chemoautotrophic oxidation of ammonium (NH<sub>4</sub><sup>+</sup>) to nitrate (NO<sub>3</sub><sup>-</sup>) carried out by two different genera of bacteria belonging to the family of *Nitrobacteraceae* (Buchanan, 1917), which can be generalised into two simple steps:

1.  $\text{NH}_3 \rightarrow \text{NO}_2^- + \text{H}^+$  (*Nitrosomonas*)
2.  $\text{NO}_2^- \rightarrow \text{NO}_3^-$  (*Nitrobacter*)

Each of the two genera (*Nitrosomonas* and *Nitrobacter*) is specialised in only one of the two oxidising steps. Nitrite ( $\text{NO}_2^-$ ) rarely accumulates in soil, as the second step generally proceeds faster than the first one. For these groups of chemoautotrophic bacteria the oxidation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$  and to  $\text{NO}_3^-$  is the sole energy source available (Wood, 1986). With the exception of some strains of *Nitrobacter*, nitrifying bacteria utilise  $\text{CO}_2$  as the major carbon source (Bock, 1978; Matin, 1978). Few strains are able to grow mixotrophically, assimilating organic compounds, but the rate of growth on these substrates is quite limited (Matin, 1978; Krummel and Harms, 1982). Nitrifiers have a very slow growth rate as, for each carbon fixed in the chemosynthesis, they have to oxidise about 35 molecules of  $\text{NH}_4^+$  or 100 molecules of  $\text{NO}_2^-$  (Baas Becking and Parks, 1927).

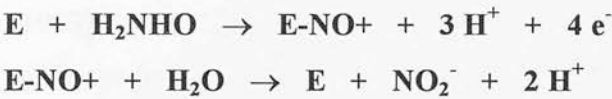
The oxidation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$  is obtained through several reactions. The first key reaction is the transformation of ammonia ( $\text{NH}_4^+$ ) to hydroxylamine ( $\text{NH}_2\text{OH}$ ), which involves  $\text{NH}_4^+$  (Suzuki *et al.*, 1974), molecular  $\text{O}_2$  (Hollocker *et al.*, 1981) and reductant (Hooper, 1969; Suzuki *et al.*, 1976) and yields hydroxylamine as a product (Lees, 1952; Hofman and Lees, 1953; Nicholas and Jones, 1960) :



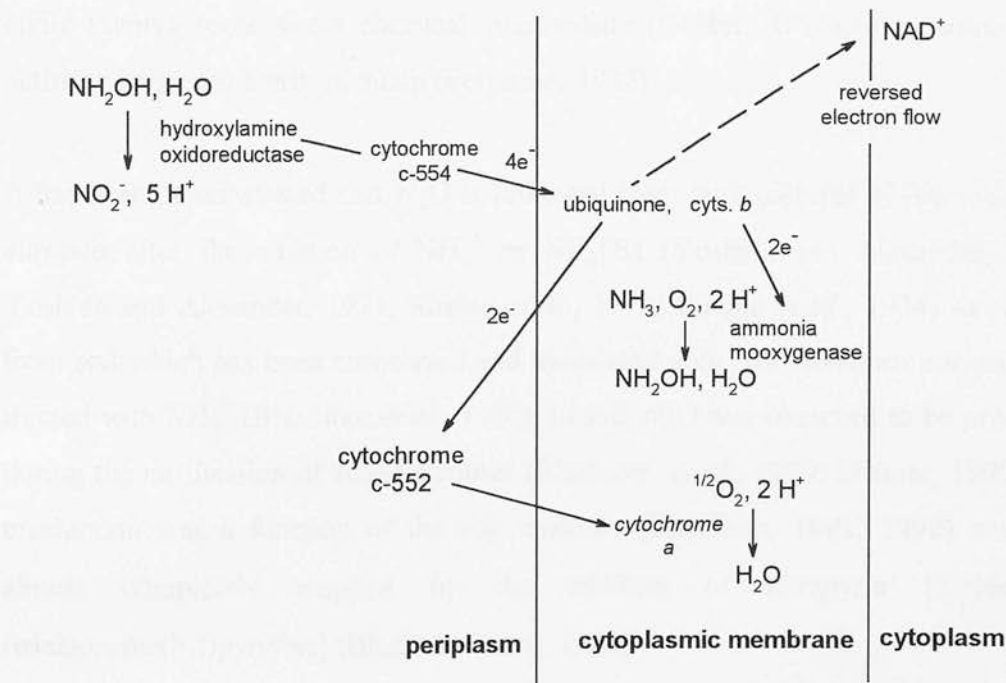
The reaction is catalysed by an enzyme known as ammonia monooxygenase, which is located in the cellular membrane (Suzuki and Kwok, 1981; Tsang and Suzuki, 1982). Acetylene ( $\text{C}_2\text{H}_2$ ) acts as an irreversible inhibitor of this enzyme (Hynes and Knowles, 1978). It seems that the enzyme, in the attempt to oxidise the  $\text{C}_2\text{H}_2$ , becomes covalently bound to the substrate and remains permanently modified and destroyed (Hyman and Wood, 1985).



The oxidation of  $\text{NH}_4^+$  to  $\text{NH}_2\text{OH}$  is energetically unfavourable ( $\Delta G^\circ = +17 \text{ kJ/mol}$ ) (Wood, 1986). For the reaction to proceed, there is the need for a parallel reaction which provides electrons. It seems that monooxygenase accepts electrons from the ubiquinone-cytochrome b region of the transport chain (see below), with NADH acting as a donor (Wood, 1986). In a second step  $\text{NH}_2\text{OH}$  is converted to  $\text{NO}_2^-$ . Andersson and Hooper (1983) have found that water contributes one oxygen to the synthesis of  $\text{NO}_2^-$  in a mechanism described as follows:



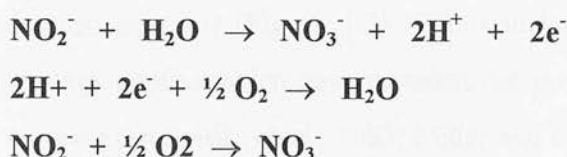
Electrons which are produced in the course of these subsequent oxidations flow through a electron transport chain, which is completely reversible except for the terminal oxidase (Figure 1.1).



**Figure 1.1** - Electron transport in *Nitrosomonas* (from Wood, 1986).

As can be seen from Figure 1.1, hydroxylamine oxidoreductase feeds electrons to a point close to the ubiquinone, while ammonia monooxygenase abstracts electrons, probably at the same point. It also can be seen that monooxygenase and terminal oxidase are competing sinks for the electrons and the balance between them needs to be carefully controlled.

Nitrite oxidation is carried out by a different bacterium, *Nitrobacter*, with detectable intermediates and the extra atom of oxygen which is derived from water (Aleem *et al.*, 1965, Kumar *et al.*, 1983):

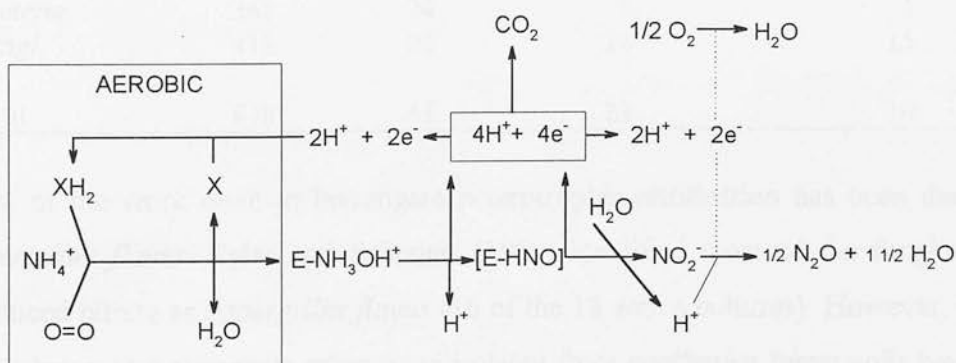


The oxidation is coupled to ATP synthesis (Cobley, 1976a) via a mechanism which could involve some direct chemical intermediate (Cobley, 1976a,b) or some other pathway involving a proton pump (Ferguson, 1982).

It has been demonstrated that  $\text{N}_2\text{O}$  is produced from pure cultures of *Nitrosomonas europaea* after the addition of  $\text{NH}_4^+$  or  $\text{NH}_2\text{OH}$  (Yoshida and Alexander, 1970; Yoshida and Alexander, 1971; Ritchie *et al.*, 1972; Ritchie *et al.*, 1974) as well as from soil which has been autoclaved and inoculated with *Nitrosomonas europaea* and treated with  $\text{NH}_4^+$  (Blackmer *et al.*, 1980). In soil,  $\text{N}_2\text{O}$  was observed to be produced during the nitrification of added fertiliser (Blackmer *et al.*, 1980; Eichner, 1990); the production was a function of the soil moisture (Davidson, 1991, 1992) and was almost completely stopped by the addition of nitrapyrin [2-chloro-6-(trichloromethyl)pyridine] (Blackmer *et al.*, 1980).

Goreau *et al.* (1980), by the examination of four genera (*Nitrosomonas*, *Nitrosolobus*, *Nitrospira* and *Nitrosococcus*), found that while the yield of  $\text{N}_2\text{O}$  was 0.1-0.5% (moles of  $\text{N}_2\text{O}$ -N per mole of  $\text{NO}_2^-$ ) under full aeration, it increased to about 10% as

the oxygen level was reduced (about 3 mg O<sub>2</sub>/litre). Investigations on chemical intermediates formed during ammonium oxidation to hydroxylamine and nitrite suggested that N<sub>2</sub>O is produced by *Nitrosomonas europaea* by a variety of mechanisms, including reduction of nitrite under aerobic and anaerobic conditions, and production of N<sub>2</sub>O during nitrification by various reactions of intermediates formed during ammonium oxidation, enhanced by oxygen stress which promotes the formation and retention of intermediates (Ritchie and Nicholas, 1972). On the basis of <sup>15</sup>N isotope tracer experiments, Poth and Focht (1985) rejected on kinetic grounds the hypothesis that N<sub>2</sub>O may be also produced by nitrification-unstable intermediates. They concluded that *N. europaea* produces N<sub>2</sub>O in conditions of oxygen stress, using nitrite as a terminal electron acceptor (Figure 1.2). This conclusions were further supported by the enzymology studies which have revealed the presence of a soluble nitrite reductase in *N. europaea* (Di Spirito *et al.*, 1985; Miller and Nicholas, 1985). Andersson and Hooper (1983), using nuclear magnetic resonance techniques, presented evidence that one of the O atoms in nitrite comes from molecular oxygen while the other comes from the water (Figure 1.2). Poth and Focht (1985) used this finding to further support the idea that “it is the initial oxidation of NH<sub>4</sub><sup>+</sup> to NH<sub>2</sub>OH, apparently catalysed by a mixed-function oxidase, that requires molecular oxygen”. The oxidation of hydroxylamine to nitrite would require no additional molecular oxygen.



**Figure 1.2** - Oxidative and reductive processes involved in N<sub>2</sub>O production by *Nitrosomonas europaea*. X is an electron acceptor, E is the enzyme portion of the intermediate. (From Poth and Focht, 1985).

The proposed mechanism is perfectly consistent with the general observation on production of  $\text{N}_2\text{O}$  by autotrophic nitrification: 1) nitrification inhibitors, blocking the production of hydroxylamine, would eliminate the source of electrons for the reduction of  $\text{NO}_2^-$  to  $\text{N}_2\text{O}$ ; 2)  $\text{N}_2\text{O}$  production is correlated with  $\text{NH}_4^+$  but not with  $\text{NO}_3^-$  additions; 3)  $\text{N}_2\text{O}$  production is directly related to oxygen stress.

#### 1.4.2 HETEROTROPHIC NITRIFICATION

Though nitrification in soil has been considered to be mainly autotrophic, the involvement of heterotrophic microorganisms in this process has been demonstrated. Eylar and Schmidt (1959) isolated 978 cultures of heterotrophic organisms from twelve actively nitrifying soils and tested them for their ability to form  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in glucose peptone broth. Though the yields of  $\text{NO}_2^-$  were very low, fungus isolates were the most numerous and active  $\text{NO}_2^-$  producers and fifteen of the fungi formed  $\text{NO}_3^-$  in addition to  $\text{NO}_2^-$  (Table 1.3).

**Table 1.3** - Microbial forms capable of producing  $\text{NO}_2^-$  and  $\text{NO}_3^-$  when grown on glucose and peptone medium (from Eylar and Schmidt., 1959).

<i>Isolates</i>	Total number	Isolates forming $\text{NO}_2^-$ -N in excess of		Isolates forming $\text{NO}_3^-$ -N in excess of
		0.2 $\mu\text{g/ml}$	0.5 $\mu\text{g/ml}$	5.0 $\mu\text{g/ml}$
<i>Actinomycetes</i>	222	16	1	0
<i>Bacteria</i>	341	24	8	1
<i>Fungi</i>	415	26	14	15
Total	978	66	23	16

Most of the work done to investigate heterotrophic nitrification has been done on *Aspergillus flavus*. Eylar and Schmidt. (1959) identified most of the fungi which produced nitrate as *Aspergillus flavus* (16 of the 18 active cultures). However, it has been shown that numerous other fungi isolated from coniferous forest soils have the ability to nitrify (Remacle, 1977a; Remacle, 1977b; Johnsrud, 1978).

Though fungi have been found to be the most efficient heterotrophic nitrifiers (Odu and Adeoye, 1970) a number of heterotrophic bacteria (Tate, 1977; Castignetti and Hollocher, 1982; Papen *et al.*, 1989) and an actinomycete (Remacle, 1977b) have been identified as potential nitrifiers in soil.

Different pathways of heterotrophic nitrification have been postulated, though there is no conclusive evidence for any of them. Aleem (1975) has given evidence for an inorganic pathway involving  $\text{NH}_2\text{OH}$  and nitrite as likely intermediates:



Evidence has also been given for some organic pathway involving oxidation of an amine or amide in place of hydroxylamine, with subsequent oxidation to a nitroso and then to a nitro-compound (Doxtader, 1965):



From the results reported in the literature it seems that heterotrophic nitrification does not yield any significant quantity of energy. The heterotrophic bacterium *Tiosphera pantotropha*, has been found able to catalyse the oxidation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$  only if an organic electron donor is present (acetate in the specific case) (Kuenen and Robertson, 1987; Robertson *et al.*, 1988). Also, other heterotrophic microorganisms have been found to be able to nitrify, but only if a source of energy is supplied (Castignetti, 1988). Nitrification led by the fungus *Aspergillus flavus* has been proposed as a mechanism which could function as an endogenous metabolism for the organism (Van Gool and Schmidt, 1973).

The literature about  $\text{N}_2\text{O}$  production by heterotrophic nitrification is very scarce. Papen *et al.* (1989) found that under aerobic conditions,  $\text{N}_2\text{O}$  production (plus  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and  $\text{NO}$ ) was detectable shortly after cultures of *Alcaligenes faecalis* (DSM 30030) started growth, and proceeded exponentially during the logarithmic growth

phase. Yoshida and Alexander (1970) found that cultures of *Aspergillus flavus* and *Penicillium atrovenetum* were able to produce significant amounts of  $N_2O$  when  $NO_2^-$  was added to the medium, while  $NO_3^-$  produced no effect. *Tsa. pantotropha* has been found to produce small quantities of  $N_2O$  during the process of heterotrophic nitrification (Robertson and Kuenen, 1988). No mechanism has been suggested, up to now, to explain the production of  $N_2O$  from this source.

#### 1.4.3 DENITRIFICATION

When oxygen concentration in the environment is not sufficient to supply for the demand of microbial respiration, some micro-organisms are able to use nitrogenous oxides as electron acceptors to carry out an anaerobic respiration. The process is generally referred as dissimilatory nitrate reduction as, starting from the form of  $NO_3^-$ , N can be reduced, in subsequent steps, until it is finally transformed into molecular N ( $N_2$ ), and can be lost from the system as NO,  $N_2O$  or  $N_2$ :



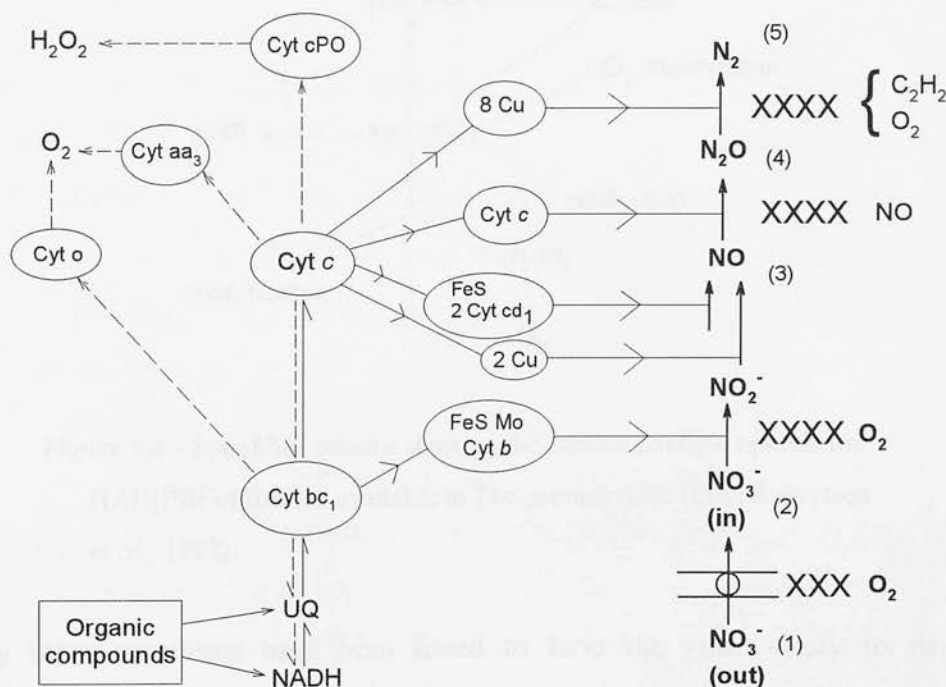
The steps of these reductions are carried out by facultative anaerobic micro-organisms, mainly heterotrophs of the genera *Pseudomonas* and *Alcaligenes* (Gamble *et al.*, 1977), which require carbon as their energy source, though several chemoautotrophic and phototrophic micro-organisms are able to denitrify as well. However, bacteria are the only microorganisms which seem to have the property of denitrification, as fungi have been found to produce  $N_2O$  from  $NO_3^-$  from some mechanism which is apparently different from the denitrification pathway (Yoshida and Alexander, 1970; Bollag and Tung, 1972). Interestingly, the property of denitrification is scarce in anaerobes or other organisms which are able to ferment.

There has been a controversy about whether NO and  $N_2O$  are intermediates in the process of denitrification. There is strong evidence that  $N_2O$  is a real intermediate for most of the denitrifiers and for the natural soil flora (Firestone *et al.*, 1979a). In contrast, it has been proposed that NO exists as an enzyme-bound intermediate, that



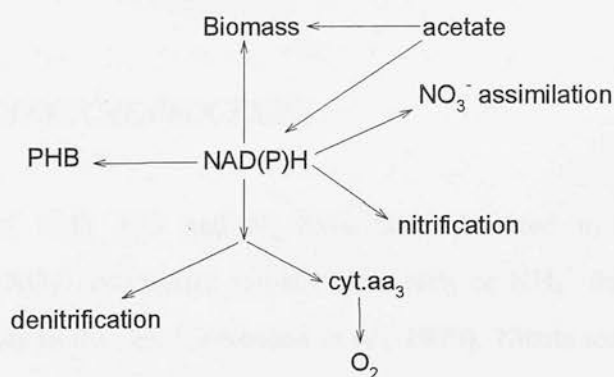
in some organisms can be exchanged with added NO (Garber and Hollocker, 1981; Firestone *et al.*, 1979b).

In the overall process (Figure 1.3)  $\text{NO}_3^-$  is transported through the cellular membrane into the cytoplasm (1). It is then reduced in 4 steps by nitrate (2), nitrite (3), nitric oxide (4) and nitrous oxide (5) reductases (Payne, 1981). Some organisms possess the overall pathway, while others may carry out only a few of these steps. The electrons necessary for these reductions are obtained from organic matter via an electron transport chain (Figure 1.3).  $\text{O}_2$  and  $\text{H}_2\text{O}_2$  can be used as an alternative electron acceptors. At each step the process can be inhibited by different substances; acetylene ( $\text{C}_2\text{H}_2$ ) at high concentrations (§ 2.5) can inhibit step (5), while oxygen can inhibit both denitrifying enzyme activity and the synthesis of new denitrifying enzymes (Payne, 1973; Smith and Tiedje, 1979).



**Figure 1.3** - The process of denitrification. Reductase redox-active centres are shown encircled. (From Lloyd, 1993).

There is much controversy about this point, however, as certain species have been found to denitrify in a range of dissolved oxygen concentration that goes from 90% of air saturation for *Thiosphera pantotropha* (Robertson and Kuenen, 1984) to 53% for *Alcaligenes sp.* (Krul and Veeningen, 1977) to complete anaerobic conditions for *Paracoccus denitrificans* (Alefounder *et al.*, 1981). Robertson *et al.* (1988) have found that *Thiosphera pantotropha* is able simultaneously to respire oxygen, to denitrify and to nitrify, the latter two metabolic pathways reaching the maximum activity at 25 % air saturation. This behaviour of *Tsa. pantotropha* has been explained as the presence of a “bottleneck” in the flow of electrons of the respiratory chain at the level of cytochrome c, which limits the rate at which NADH can be reoxidized by this route. This causes a reduction in the cytochrome chain which allows electrons to flow to other pathways (Figure 1.4), thus allowing a faster rate of NADH oxidation (Robertson *et al.*, 1988).



**Figure 1.4** - Simplified scheme showing the various possible options for NAD(P)H utilization available to *Tsa. pantotropha* (from Robertson *et al.*, 1988).

Many other organisms have been found to have the same ability to denitrify aerobically, often associated with heterotrophic nitrification activity (for a review see Robertson and Kuenen, 1990b).

Denitrification activity has been reported in dried soils and in desert soils (Virginia, 1982; Smith and Parsons, 1985; Peterjohn, 1991), where it seems to depend on a



complex interplay between soil moisture, carbon, nitrogen availability, pH, temperature and  $O_2$ . It is still a matter of controversy whether the combinations of environmental factors can create microsites convenient for denitrification activity in aerobic soils, or if aerobic denitrification does really occur (Lloyd, 1993).

#### 1.4.4 OTHER BIOLOGICAL PROCESSES

Nitrous oxide has been reported to be produced by a number of  $NO_2^-$  and  $NO_3^-$  reducing bacteria common in soil (species of *Bacillus*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Escherchia*, *Erwina*), which do not fit the definition of denitrifiers as given above (Tiedje, 1981; Smith and Zimmerman, 1981; Anderson and Levine, 1986).  $NO_2^-$  or  $NH_4^+$  were the major products of  $NO_3^-$  reductions by these non-denitrifying bacteria, and less than 30% of the reduced  $NO_3^-$  was released as  $N_2O$ . The ecological significance of these processes on the overall  $N_2O$  fluxes in soil is unknown.

#### 1.4.5 NON-BIOLOGICAL PROCESSES

Gaseous losses of  $N_2O$ ,  $NO$  and  $N_2$  have been detected in soils where high concentrations of  $NO_2^-$  accumulate, usually after  $NH_3$  or  $NH_4^+$  fertilizers have been applied at high rates to the soil (Stevenson *et al.*, 1970). Nitrite ion, in acid medium, reacts chemically with organic molecules forming unstable nitroso compounds ( $-N=O$ ), which can form easily gaseous products (Bremner, 1957). This process is defined as chemodenitrification and occurs mainly in acid soils with high concentrations of organic material (Nelson, 1982). However, even in neutral soils receiving high levels of  $NH_4^+$  fertilizer, soil microsites, where nitrification creates high concentrations of  $NO_2^-$  and  $H^+$ , could have optimal conditions for chemodenitrification losses of gaseous N.

$N_2O$  can also be formed in reactions between  $NO_3^-/NO_2^-$  and some inorganic compounds such as  $Fe^{2+}$  or  $Cu^{2+}$ . Those reactions could be particularly important in ground waters (Van Hecke *et al.*, 1990).

## 1.5 FACTORS CONTROLLING N<sub>2</sub>O FLUXES IN SOIL

### 1.5.1 SOIL AERATION

Though the influence of soil aeration on N<sub>2</sub>O emissions is quite complicated, depending on the interaction of several factors, it is commonly accepted that N<sub>2</sub>O emissions increase as aeration becomes restricted (Dowdell and Smith, 1974; Egginton and Smith, 1986).

An inverse relationship between the rate of denitrification and O<sub>2</sub> concentration has been demonstrated in several studies (Parkin and Tiedje, 1984; Burton and Beauchamp, 1985; Arah *et al.*, 1991). This relationship has been found to be more evident with increasing temperature (Focht, 1974). The composition and quantity of the products of denitrification are both influenced by O<sub>2</sub> partial pressure in soil. O<sub>2</sub> delays the synthesis of nitrous oxide reductase relative to nitrate and nitrite reductase, so that the ratio N<sub>2</sub>O/N<sub>2</sub> increases with increasing oxygen concentrations (Focht, 1974; Smith *et al.*, 1983; Bonin *et al.*, 1989). However, as O<sub>2</sub> concentration in soil becomes almost zero, as after flooding a soil, the major product of denitrification becomes N<sub>2</sub>, as N<sub>2</sub>O is used as an electron acceptor in the extremely reduced environment (Terry *et al.*, 1981; Mosier *et al.*, 1990).

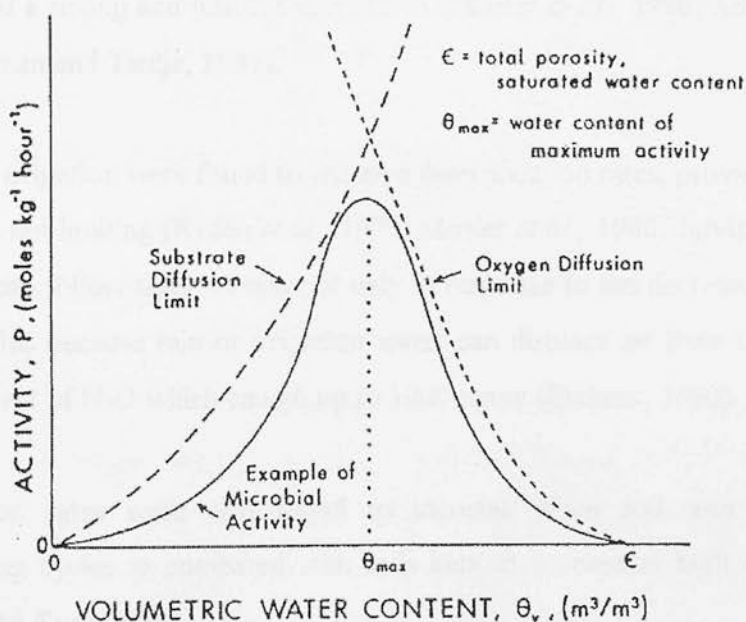
Production of N<sub>2</sub>O by nitrification has been found to increase with decreasing O<sub>2</sub> concentration, either in pure cultures of *Nitrosomonas* sp. (Goreau *et al.*, 1980) or in soil samples amended with ammonium and exposed to an atmosphere with increasing concentration of CO<sub>2</sub> (Keeney *et al.*, 1985). In none of the cases, however, did a total lack of oxygen promote N<sub>2</sub>O production from nitrification.

Diffusion of oxygen in soil depends on the texture, management, water content, consumption by microbial process and consequently availability of oxidizable compounds (Smith, 1990) (more details are given in the following paragraphs). Due to the complex interaction of those factors it has been found that alternate anaerobic-aerobic cycling increases N<sub>2</sub>O evolution by a factor of 10 to 20 relative to constant aerobic conditions (Smith and Patrick, 1983).

### 1.5.2 SOIL WATER STATUS

Soil water content regulates nitrification and denitrification mainly by two distinct mechanisms. The first is the possibility of diffusion, through the water films, of the substrates  $\text{NH}_4^+$  and  $\text{NO}_2^-$ , for nitrifying bacteria, and  $\text{NO}_3^-$  and  $\text{NO}_2^-$ , for denitrifying bacteria; while the second is the role of the water in soil pores as a controller of gaseous diffusion within the soil atmosphere (Smith, 1990; Davidson, 1991).  $\text{O}_2$  diffuses about 10,000 times slower in the water than in the air, so that the amount of water filling the soil pores, through which the oxygen movement occurs, affects the flux of  $\text{O}_2$  into the soil, as well as the flux of gases as  $\text{N}_2\text{O}$  or  $\text{CO}_2$  out of the soil.

Skopp *et al.* (1990) predicted the diffusion of substrate in soil solution as a function of the water filled pore space (WFPS), and found that aerobic processes have their optimum at about 60% WFPS, which represents the intersection of increasing availability of organic-C and inorganic-N and decreasing availability of  $\text{O}_2$ , with increasing water content (Figure 1.5).



**Figure 1.5** - Conceptual plot of microbial activity as a function of soil water content.  $P$  stands for the rate of production for each microbial process. (From Skopp *et al.*, 1990).

At low water content  $\text{N}_2\text{O}$  emissions are low because microbial activity is low and  $\text{O}_2$  is largely available; nitrification then produces mostly  $\text{NO}_3^-$ . With increasing water content, mineralization increases and nitrification increasingly produces  $\text{N}_2\text{O}$  by reduction of  $\text{NO}_2^-$  in the presence of a limited concentration of oxygen (see § 1.4.1). For WFPS in the range of 30% to 70% nitrification has been found positively correlated to the soil water content (Freney *et al.*, 1979; Goodroad and Keeney, 1984; Davidson *et al.*, 1993).

Denitrification rate increases with increasing WFPS (i.e. decreasing  $\text{O}_2$ ), peaking at 100% WFPS. However,  $\text{N}_2\text{O}$  production by denitrifiers peaks at 80-90% WFPS (Linn and Doran, 1984), because for a further increase of WFPS (> 80%) an increasing proportion of  $\text{N}_2\text{O}$  produced is reduced to  $\text{N}_2$  (Figure 1.6).

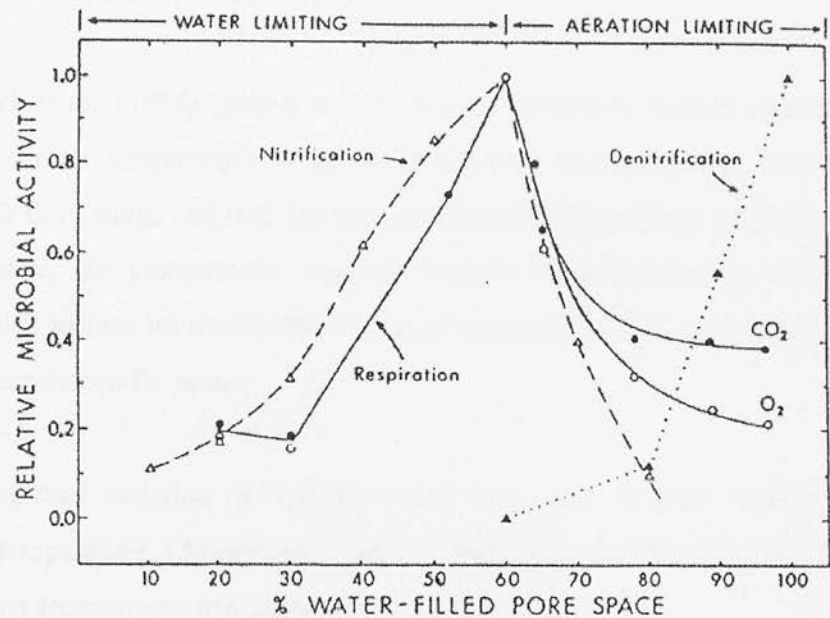
Though some authors have found a weak correlation between soil water content and the rate of denitrification (Limmer and Steele, 1982; Hixson *et al.*, 1990), most demonstrated a strong and positive correlation (Mosier *et al.*, 1986; Smith and Arah, 1990; Groffman and Tiedje, 1991).

Rainfall and irrigation were found to increase denitrification rates, provided that other factors were not limiting (Ryden *et al.*, 1979; Mosier *et al.*, 1986; Jarvis *et al.*, 1991).  $\text{N}_2\text{O}$  peaks can follow those events not only in response to the decreased  $\text{O}_2$  content in soil but also because rain or irrigation water can displace air from the soil pores, with its content of  $\text{N}_2\text{O}$  which can go up to 1000 ppmv (Eichner, 1990).

Denitrification rates were also found to increase when soil was subjected to wetting/drying cycles as compared with soils kept at a constant high water content (Mulvaley and Kurtz, 1984).

Moreover, the rates of denitrification have not been found to be the same for a certain water content, when this water content is obtained by drying or wetting the soil. The denitrification rate increases much more sharply when wetting soils at very

low water content than it declines when soils are dried to very low water content (Groffman and Tiedje, 1988).



**Figure 1.6** - The relation between water-filled pore space (% WFPS) and relative amounts of nitrification, denitrification, O<sub>2</sub> uptake by microbial respiration and CO<sub>2</sub> production by microbial respiration. (From Linn and Doran, 1984).

### 1.5.3 TEMPERATURE

Laboratory studies on nitrification and denitrification have demonstrated that N<sub>2</sub>O production rate increases strongly with increasing temperature up to 20-40 °C (Freney *et al.*, 1979; Keeney *et al.*, 1979; Goodroad and Keeney 1984). In the field, low rates of denitrification have been reported even at temperatures just below zero (Malhi *et al.*, 1990; Dorland and Beuchamp, 1991), though higher temperatures (> 5 °C) are required to produce significant denitrification rates (Vinther, 1990, cited in Granli and Bøckman, 1994). The optimum temperature for denitrification activity ranges from 30 to 67 °C and denitrification stops around 70°C (Keeney *et al.*, 1979; Malhi *et al.*, 1990). As temperature increases the ratio N<sub>2</sub>O/N<sub>2</sub> decreases (Keeney *et al.*, 1979).

The optimum temperature for nitrification is slightly lower, 25-35 °C (Bock *et al.*, 1986). Also, indigenous nitrifiers have been found to be able to nitrify at low temperatures, and N<sub>2</sub>O production has been found to increase for increasing temperature (Goodroad and Keeney, 1984).

Though Conrad *et al.* (1983) have found N<sub>2</sub>O daily emissions from a lawn to be related to soil surface temperature by an Arrhenius kind of relationship, Smith and Dowdell (1974) have suggested that the relationship with temperature could be even more complicated, as temperature controls oxygen consumption by microbial respiration, which in turn influences the extent of anaerobic zones, where processes such as denitrification could occur.

Diurnal and seasonal variation in N<sub>2</sub>O emissions have been in part explained by variations of temperature (Armstrong, 1983; Christensen, 1983). Usually fluxes increase at higher temperature and higher fertilizer applications.

#### 1.5.4 SOIL ORGANIC MATTER

N<sub>2</sub>O production has been reported to be positively correlated with soil organic matter by several authors (Bremner and Blackmer, 1981; Robertson and Tiedje, 1984). When organic manure was applied with mineral N fertilizer the N<sub>2</sub>O produced has been found to be much higher than the N<sub>2</sub>O generated by the sole mineral N addition (Christensen, 1983; Bouwman, 1990). However, the ratio C/N of the manure is important. Bremner and Blackmer (1981) reported that the N<sub>2</sub>O emissions increased with decreasing C/N ratio in organic amendments. Goodroad *et al.* (1984) showed that residues of manure or alfalfa were much more effective in increasing N<sub>2</sub>O fluxes than the addition of sewage sludge or straw.

The organic material in soil stimulates microbial respiration, inducing O<sub>2</sub> consumption. When the O<sub>2</sub> consumption is faster than the O<sub>2</sub> diffusion from the air through the soil pores, anoxic conditions can arise. In these cases, a very favourable



condition for denitrification is created because reducing agents (electron donors) and low O<sub>2</sub> partial pressures are present (Parkin, 1987).

Nitrification, on the contrary, could be less favoured in the presence of fresh organic material, because heterotrophic microorganisms could compete better than nitrifiers for NH<sub>4</sub><sup>+</sup> present in the soil. Moreover, high respiration rates and low oxygen partial pressure could inhibit nitrification in favour of the denitrification process. However as organic matter is also a source of NH<sub>4</sub><sup>+</sup>, microsites heterogeneity of mineralization, and distribution of organisms and roots, can account for significant nitrification rates (Jackson *et al.*, 1989).

### 1.5.5 NITROGEN AVAILABILITY TO SOIL MICROORGANISMS

#### 1.5.5.1 N mineralization/immobilization and availability to the system

*Mineralization* and *immobilization* are the key processes which control the flux of nitrogen through the soil system.

*Mineralization* is the process led by soil microorganisms during which organic-N is released in the form of NH<sub>3</sub> or NH<sub>4</sub><sup>+</sup> and organic C in the form of CO<sub>2</sub>, while microorganisms are breaking down the organic matter to obtain nutrients and energy. Though it is carried out by most of the soil heterotrophic microorganisms, the more complex the substrate gets, the more specialized are the microorganisms which mineralize it (Killham, 1994). Rate and yield of mineralization depend on several factors such as quality of the substrate, temperature, water content and pH of the soil (Stanford *et al.*, 1973; Linn and Doran, 1984).

Mineralization is always accompanied by *immobilization*. Most of the N released during mineralization is quickly assimilated in the microbial biomass and immobilized in the microbial tissue, if enough C is available. Fungi generally have a higher C/N ratio than bacteria and may therefore immobilize less N per unit of substrate than



bacteria. However this difference can be offset by fungi having a higher efficiency for C assimilation (less C lost as CO<sub>2</sub>) (Wood, 1995). The immobilized N will be newly available at the end of the growing phase of the microbial population, as the turnover of the microbial biomass is very fast (few days) and the microbial N one of the main components of the “available N pool” in the soil.

The difference between gross mineralization and gross immobilization is the *net mineralization*. One of the critical points in the net balance mineralization/immobilization is the C/N ratio of the substrate. To be able to measure a net mineralization, it is usually necessary that the substrate to be decomposed has a C:N ratio lower than 30 (more than 1.18 % of N), however a generalization is very difficult to make, as other factors such as the quality of the substrate (lignin and pholiphenolic content) can influence the mineralization rates (Sequi, 1989a).

The simple monitoring of pools sizes with time, and net rates and effects, is not sufficient to describe the soil processes of mineralization and immobilization. A low net rate of mineralization may reflect either a low gross rate of mineralization or a higher rate of gross mineralization balanced by a high rate of gross immobilization. The dynamics of the soil biomass will be very different in the two cases (Jansson and Persson, 1982). A more suitable tool for determining gross and net rates of microbial processes such as mineralization, immobilization, nitrification, is represented by *isotopic techniques* (see chapter 6).

Particularly important in the process of breakdown of the organic matter are the extracellular enzymes, which persist in the soil after the microbial population has died (Burns, 1982). The extra-cellular enzymes are mainly derived from soil microorganisms, which utilise them to break down insoluble substrates which are too large to enter the cell. Plants and some animals are also able to produce such enzymes. Being themselves proteins, extracellular enzymes are also liable to degradation, hence they are usually associated in *matrix-organic matter-enzyme* complexes which protect them, at least partially, from microbial attack (Sequi,

1989b). Some extra-cellular enzymes include: *carbohydrases* such as cellulases, inducible enzymes mainly produced by fungi; esterases, such as phosphatases or nucleases; *proteases* and *amidases*, which are important in the degradation of proteins and hence in the N mineralization and in the decomposition of urea (ureases); *oxidoreductases*, such as the ligninolases produced by fungi, though some oxidoreductases such as dehydrogenases can be also intracellular.

#### 1.5.5.2 Bacterial and fungal uptake of different nitrogen forms

Bacteria: they are able to retain  $\text{NH}_4^+$  intracellularly at concentrations higher than in the culture medium (Droz *et al.*, 1972). This implies a membrane impermeable to  $\text{NH}_4^+$  and a transport system for the uptake of the ion. Bacteria can also take up amino acids directly as a source of N and C, utilising active transport systems which have strict specificities and are of high physiological activity. This serves a dual role in the cell physiology: it is a chemical sensing system for detecting nutritional information in the external medium and provides a functional barrier for maintaining pool amino acids in the cell (Ankaru, 1978, Rosen and Lashket, 1978). If amino acids are bound in the form of peptides they can be either transported in the cell through peptides permeases and then hydrolysed, or they can be hydrolysed outside the cell, in the periplasmic space and then the amino acids can be taken up (Payne, 1980). Bacteria are unable to take up proteins so they secrete proteinases outside the cell membrane, which are highly specific (Law, 1980). It is not clear how induction of proteinases operates.

Bacteria have also been found to be able to assimilate  $\text{NO}_3^-$ . This is a highly regulated process which generally proceeds slowly at the rate at which  $\text{NH}_4^+$  is required for growth (Wood, 1995). Indeed,  $\text{NH}_4^+$  rather than  $\text{NO}_3^-$  is the preferred N source by microorganisms in the immobilization reactions in soil (Rice and Tiedje, 1989).

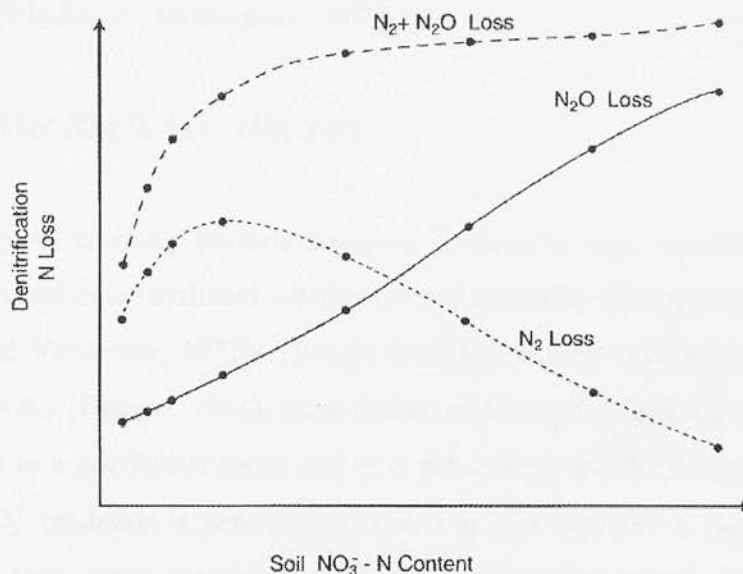
Fungi: these organisms can utilise  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$ .  $\text{NO}_3^-$  and  $\text{NO}_2^-$  will generally induce the synthesis of the enzymes necessary for the assimilatory reduction of nitrogenous compounds; however, reduced forms of nitrogen (e.g.  $\text{NH}_4^+$  or

glutamine) will act as repressors of such a mechanism (Tomsett, 1988).  $\text{NH}_4^+$  is uptaken actively. The uptake system is induced when substrate is added after a phase of starvation and cycloheximide has been found to inhibit the development of the uptake system (Pateman *et al.*, 1973). Intracellular concentrations of  $\text{NH}_4^+$  control the  $\text{NH}_4^+$  uptake by repression of the synthesis of some protein components. Fungi, as well as bacteria, are able to uptake amino acids, however their transport system can vary from highly specific to less specific, as for *Aspergillus nidulans* which possesses four or five transport systems for all the amino acids (Piotrowska *et al.*, 1976). One characteristic of amino acids transport in fungi appears to be the ability of the ammonium ion to repress the activity on formation of individual amino acids transport systems (Pateman *et al.*, 1973). As for the bacteria the transport system is “active” and binding-proteins mediated (Wolfenbarger, 1980). Fungi can utilise peptides and proteins, generally secreting extra-cellular proteases and peptido-hydrolases which break down proteins to monomer amino acids or to peptides smaller than hexapeptides. These are later transported into the cell and hydrolysed within it or during the transport through the membrane. The liberated amino acids dictate the continued synthesis and/or release of extra-cellular peptido-hydrolytic activity (Wolfenbarger L., 1980).

#### 1.5.5.3 *N availability and $\text{N}_2\text{O}$ production*

Availability of mineral N ( $\text{NH}_4^+$  and  $\text{NO}_3^-$ ) to microorganisms is an important controller for the processes which produce  $\text{N}_2\text{O}$ .  $\text{NH}_4^+$  is usually limited in soil, as the oxidation of  $\text{NH}_4^+$  generally proceeds faster than  $\text{NH}_4^+$  production through mineralization. An exception is the practice of ploughing in crop residues rich in N, such as legumes, which are easily mineralizable (Duxbury *et al.*, 1982).  $\text{NO}_2^-$  is very rapidly oxidized and does not accumulate significantly in soil (MacDonald, 1986).

Denitrification rate generally increases with increasing  $\text{NO}_3^-$  concentration (Ryden, 1983; Robertson *et al.*, 1987; Ambus and Lowrance, 1991), but then reaches a plateau (Mosier *et al.*, 1983; Figure 1.7).



**Figure 1.7** - The idealized effect of soil  $\text{NO}_3^-$  on  $\text{N}_2$  and  $\text{N}_2\text{O}$  losses associated with denitrification. (From Mosier *et al.*, 1983).

Limmer and Steele (1982) have found denitrification potential to be independent from  $\text{NO}_3^-$  concentration for values greater than  $25 \text{ mg NO}_3^- \text{ -N kg}^{-1}$  in a range of soils. Moreover, when other factors are limiting, denitrification can be rather insensitive to variation in  $\text{NO}_3^-$  concentration (Kroeze *et al.*, 1989; Aulakh *et al.*, 1983, Bremner, 1978).

The  $\text{N}_2\text{O}/\text{N}_2$  ratio strongly increases with increasing  $\text{NO}_3^-$  concentrations (Figure 1.7) as high  $[\text{NO}_3^-]$  inhibits  $\text{N}_2\text{O}$  reduction to  $\text{N}_2$  (Blackmer and Bremner, 1978; Firestone *et al.*, 1980; Kroeze *et al.*, 1989). It is not clear if this is a true inhibition of  $\text{N}_2\text{O}$  reduction or if it is due to the greater suitability of  $\text{NO}_3^-$  as an electron acceptor as compared with  $\text{N}_2\text{O}$  (Cho and Sakdian, 1978). In this latter case, the effect of nitrate is nullified when soils are strongly reduced, as after a flood (Terry and Tate, 1980; Bowman, 1990). Firestone *et al.* (1980) proposed that the inhibitory effect of  $\text{NO}_3^-$  on  $\text{N}_2\text{O}$  production could be actually due to  $\text{NO}_2^-$ , which is much more effective in such an inhibition than  $\text{NO}_3^-$ , and which could be produced by microbial processes when high doses of  $\text{NO}_3^-$  are applied to the soil. Such an inhibitory effect of  $\text{NO}_2^-$  on  $\text{N}_2\text{O}$  reduction has been found also by Van Cleemput *et al.* (1988). Low concentrations of

$\text{NO}_3^-$  (5 mg N kg soil<sup>-1</sup>) have been found to stimulate formation and activity of nitrous oxide reductase (Blackmer and Bremner, 1979).

#### 1.5.6 CHEMICAL STATUS OF THE SOIL

Both denitrifying and nitrifying bacteria are quite sensitive to high concentrations of hydrogen ions and have an optimum activity around neutrality (Bremner and Shaw, 1958b; Focht and Verstraete, 1977). Though nitrifying activity have been found to stop at pH below 4.5 (Duggin, 1991), some strains of *Nitrospira* have been found to be able to nitrify in a coniferous forest soil at a pH around 4 (Martikainen and De Boer, 1993).  $\text{NO}_2^-$  oxidation is generally restricted at pHs between 5 and 8, being  $\text{NH}_3$  and  $\text{HNO}_2$  toxic when present in alkaline or acid environments, respectively (Focht and Verstraete, 1977). Heterotrophic nitrification is considered to be the predominant process in acid environments (Killham, 1986, Duggin, 1991).

Denitrification rates were found to increase 2 to 3-fold when an acid soil was limed, increasing the pH from about 3 to more than 7 (Weier and Gilliam, 1986). The reduction of  $\text{N}_2\text{O}$ , however, was much more sensitive than the reduction of  $\text{NO}_3^-$  to acid conditions. The ratio  $\text{N}_2\text{O}/\text{N}_2$  strongly decreased with increasing pH. Frequently  $\text{N}_2\text{O}$  comprises more than half of the nitrogenous gases evolved from acid environments.

#### 1.5.7 SOIL TEXTURE

Clay soils can hold more water than drained sandy soils and consequently they have a higher potential for  $\text{N}_2\text{O}$  production by denitrification. However,  $\text{N}_2\text{O}$  can escape more easily from coarse-textured soil (Granli and Bøckman, 1994). When clay soils are very wet, the diffusion of  $\text{N}_2\text{O}$  from depth to surface is very slow and the possibility that  $\text{N}_2\text{O}$  is reduced to  $\text{N}_2$  is much higher (Arah *et al.*, 1991). On the contrary the aerobic process of nitrification is favoured in the lighter textured soil. In general, it has been found that  $\text{N}_2\text{O}$  produced from fine textured soils is significantly

higher than  $\text{N}_2\text{O}$  produced in more coarse textured soils (McKenney *et al.*, 1980; Matson *et al.*, 1990; Skiba *et al.*, 1992).

The size of soil aggregates is also important, as bigger aggregates will become more easily anaerobic but organic and mineral substrate will diffuse into them less easily. Arah and Smith (1989) included aggregate size in a denitrification model, indicating soil texture, structure and water content as the main factors affecting denitrification rates. Denitrification rate increased strongly, as a consequence of  $\text{O}_2$  limitation, as the aggregate size increased from 5 to 30 mm.

#### 1.5.8 LAND USE

Land use change contributes to  $\text{N}_2\text{O}$  emissions. Conversion of forests and grasslands to arable land or pasture accelerates C and N cycling, causing increased  $\text{N}_2\text{O}$  emissions (Vitousek and Matson, 1990). The conversion, over the past 100 years, of about  $8.5 \times 10^8$  ha of natural land into cultivated land, has been estimated to have released about 0.2 - 0.6 Tg of  $\text{N}_2\text{O}$  (Vinten and Smith, 1993).

Ploughing and cultivation usually increases soil aeration, enhance moisture evaporation, and increase the contact between crop residues and microorganisms. While this has been found to enhance the  $\text{N}_2\text{O}$  emissions derived from the nitrification process, it has been generally observed that denitrification and  $\text{N}_2\text{O}$  losses are higher from undisturbed than from ploughed soils (Linn and Doran, 1984; Staley *et al.*, 1990).

Compaction caused by tractor traffic can increase both denitrification (Bakken *et al.*, 1987; Torbert and Wood, 1992) and  $\text{N}_2\text{O}$  emission rates (Hansen *et al.*, 1993) by a factor from 1.5 to 6.

Crops have a complicated influence on  $\text{N}_2\text{O}$  production. On the one hand they deplete inorganic N, but on the other hand they create available fresh organic matter as



organic exudates from the roots; moreover the respiration of the same roots can further reduce the  $O_2$  partial pressure in the rhizosphere. The net effect of these influences can be very variable, depending on other soil parameters, climate, agricultural practices (in arable soils), etc.

## 1.6 OBJECTIVE OF THE RESEARCH

The present work studied  $N_2O$  emissions from two light textured Scottish soils and investigated the potential contribution of different biological processes to  $N_2O$  fluxes.

$N_2O$  is generally considered to derive mainly from the bacterial processes of autotrophic nitrification or anaerobic denitrification, however, as discussed in § 1.4, it is not yet clear what are the physiological limits and versatility of the relevant organisms. Moreover, it seems that there are many other microorganisms which are able to produce  $N_2O$  (§ 1.4) though the pathways of such production are not yet sufficiently understood. Outstanding questions include the role of aerobic denitrifiers, the capacity of autotrophic nitrifiers to produce  $N_2O$  and  $N_2$  in almost at very low oxygen tensions, the importance of heterotrophic versus autotrophic nitrification, the role of fungi in  $N_2O$  production from organic or inorganic N sources, etc.

There is plenty of literature where fluxes from different environments are reported, and, based on the correlation of these fluxes with environmental parameters ( $NH_4^+$  versus  $NO_3^-$ , aerobicity versus anaerobicity, etc.),  $N_2O$  fluxes are attributed mainly to autotrophic nitrifying bacteria (i.e. *Nitrosomonas*) or to strictly anaerobic denitrifiers. Under natural conditions, which are limiting for microbial processes most of the time, the competition for the limited resources is high and mixotrophy, or metabolic flexibility and reactivity in general, may constitute the rule rather than the exception. The possibility that processes other than autotrophic bacterial nitrification and strictly anaerobic denitrification can be involved in  $N_2O$  fluxes is something which deserves more attention.



The study was focused on light textured and well-aerated soils for two reasons: they are very widespread in Scotland and they are not studied as much as heavier soils in relation to N<sub>2</sub>O fluxes.

In Scotland, light soils cover a significant area of the country, about 20% of the whole land surface (Soil Survey of Scotland, 1984). In light textured soils, autotrophic bacterial nitrification is considered the main process which produces N<sub>2</sub>O (Bremner and Blackmer, 1978; Duxbury and McConnaughey, 1986), in contrast to heavy textured soils where denitrification is generally considered the dominant source (Smith, 1990, Clayton *et al.*, in press). As N<sub>2</sub>O emitted through nitrification is generally estimated to be less than N<sub>2</sub>O lost via denitrification, heavy soils are regarded as a more important source of N<sub>2</sub>O and studies on N<sub>2</sub>O fluxes are much more concentrated on these kind of soils rather than on light textured soils. However, because sandy and light soils are very widespread world-wide, their contribution to the total N<sub>2</sub>O flux could be of the same order of magnitude as that of heavy soils.

Both the soils studied belong to the same series, and were sampled from a woodland and from an adjacent area of arable land that had been cropped with winter wheat. It was possible therefore to compare results from two environments experiencing different land uses but having soils which did not differ too much in basic characteristics.

The research focused on the following objectives:

- To understand what are the main environmental factors which could influence N<sub>2</sub>O emissions in the two fields.
- To identify the possible biological sources of N<sub>2</sub>O.
- To try to make an approximate estimation of the relative contribution of those sources to the overall N<sub>2</sub>O flux and relate it to the field measurements.

## FIELD AND LABORATORY METHODS

### 2.1 SITE DESCRIPTION

Two adjacent sites were chosen for the present research, both located in East Lothian, at Gullane, about 30 km east-north east of Edinburgh (GR 484813). Both soils belong to the Peffer Series, an imperfectly drained brown forest soil derived from fine beach sand. One site was a winter wheat field (**A**), the other was a nearby mixed deciduous woodland (**W**) (*Ulmus sp.*, *Fagus sylvatica*, *Fraxinus excelsior sp.*, *Acer platanoides*, *Crataegus monigina*). Some soil characteristics are indicated in Table 2.1.

**Table 2.1** - Some soil characteristics of the two Gullane sites at 3 different depths.

	Soil texture	pH (H <sub>2</sub> O)	Bulk density	Total C (%)	Total N (%)	C/N
<b>Wheat field:</b>	loamy sand					
10 cm depth		8.2	1.32	1.19	0.12	9.5
20 cm "		-	-	1.02	0.12	8.8
30 cm "		-	-	1.22	0.13	9.4
<b>Woodland:</b>	sandy loam					
10 cm depth		8.0	1.15	3.24	0.31	10.3
20 cm "		-	-	2.50	0.25	9.8
30 cm "		-	-	1.61	0.17	9.5

## 2.2 GAS FLUX MEASUREMENTS

The air present in the soil pores is continuously modified in its composition, with respect to the atmospheric air composition, by microbial processes and by the activity of plant roots and soil organisms. Due to the different concentrations of gases in the soil and in the air, gases diffuse continuously between the soil and the atmosphere. The first analysis of free air in soil was done by Boussingault and Lewy (1853), by inserting a pipe into the soil (0.3-0.4 m) and aspiring 2.5-10 l of air through a barium hydroxide solution and determining the CO<sub>2</sub> present in the air by the carbonate which was formed. Since then, techniques for sampling and measuring gas concentration and fluxes in the soil have enormously improved and are continuously refined.

### 2.2.1 *FIELD SAMPLING AND MEASUREMENTS: AN OVERVIEW*

There are several methods to measure gas fluxes in the field, among them the most used are: aircraft-based techniques, tower-based micrometeorological techniques, open-path infra-red techniques, and open and closed chambers. They differ in the sensitivity of the method, the scale and the uniformity of the studied area, the time scale of the measurements, and the cost and the expertise involved, so that each of these techniques can be suitable for different purposes.

Micrometeorological techniques have several variants, including the flux gradient, eddy correlation, eddy accumulation and mass balance method (Denmead, 1983; Fowler and Duyzer, 1989, Mosier, 1990). Their advantages include the possibility of measuring over a large area (up to  $10^4 - 10^5 \text{ m}^2$ ), the possibility of avoiding a disturbance of the vegetation and the soil of the studied area, and a high frequency of sampling which makes it possible to follow changes in the rates of gas exchange. On the other hand, an extensive and uniform sampling area is required upwind of the detector and it is necessary to have constant atmospheric conditions during each measurement period.

The aircraft method offers the possibility of making measurements over large areas and is suitable for remote studies, however it is quite cost effective (Desjardins and MacPherson, 1989).

Long-path infrared monitors and Fourier transform infrared (FTIR) spectrometer systems can be used to measure the average gas concentrations over distances of ten to hundreds of metres either in the open air or under an enclosure of the order of 50 m<sup>2</sup> in size (Galle *et al.*, 1994; Smith *et al.*, 1994). FTIR is also used for the gradient micrometeorological method (Galle *et al.*, 1994). As compared with micrometeorological techniques, large enclosure IR techniques offer the advantage of being less complicated, and allow the possibility of comparing differently treated adjacent field plots, which could reciprocally interfere if micrometeorological techniques are used.

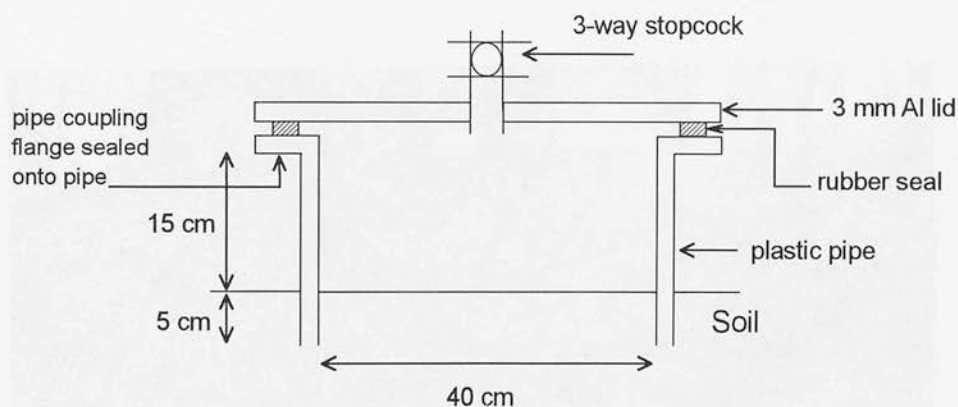
The most commonly used method for the measurements of gas fluxes is the closed chamber method (e.g. Mosier *et al.*, 1991; Smith *et al.*, 1995). This method involves the use of open-bottom chambers, which are inserted into the soil surface and closed and the accumulation of the gas measured. The disadvantage of this method consists of the exclusion of the natural turbulence of the air, which contributes to soil-atmosphere gas exchanges, and the alteration of temperature and humidity in the chamber during the sampling period.

The former problem can be in part avoided by using an internal fan or using "open chambers". In the latter system the boxes are flushed with ambient air at a constant flow rate during the measuring period and relative humidity and mixing ratios are almost at ambient conditions (Schütz and Seiler, 1989). However, a highly sensitive gas analyser is required, to measure the increase above ambient concentration in the flowing air stream. An alternative to analysis of a continuous air stream is to adsorb the gas on a molecular sieve and later to release it into a container for the analysis (Egginton and Smith, 1986).

## 2.2.2 CHAMBERS USED IN THIS PROJECT

Two kinds of closed chambers, both designed in the Soil Science Department of the Scottish Agricultural College, Edinburgh, were used for experiments in the present work. Gas samples taken with both chambers were analyzed by gas chromatography (§ 2.4).

The first kind was a “manual chamber” (Smith *et al.*, 1995). This chamber consisted of a polypropylene vent pipe (20 cm length for 40 cm diameter) fitted with a 4.5 cm wide outward facing polyvinylchloride (PVC) flange at one end (Figure 2.1).



**Figure 2.1** - Cylindrical closed chamber (Smith *et al.*, 1995).

The cylinder is inserted in the soil to about 5 cm depth in a slot previously formed by a metal cutting ring. The lid is a 3 mm thick aluminium sheet ( $49 \times 49 \text{ cm}^2$ ), provided with a circle of hollow-section rubber draught excluder on its underside. The lid is held in place on the flange by 4 clips made from short lengths of square-section PVC electric cable ducting. The lid is provided with a sampling port closed by a three-way tap.

To measure the gas flux, the lid is fixed in place, and the change in gas concentration in the chamber with time, is determined by taking replicate gas samples (1-30 ml)



from the chamber headspace by syringe (either a plastic or greased-glass syringe fitted with a 3-way tap) and transferring them to the laboratory for gas chromatographic analysis.

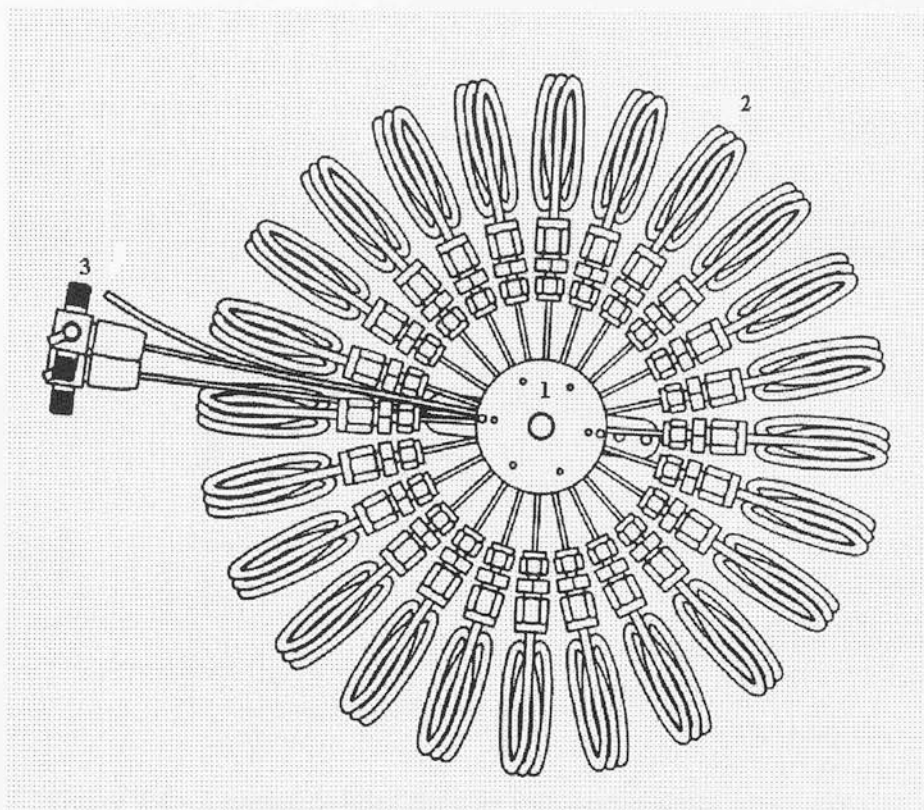
For the  $N_2O$  measurements, gas samples are taken normally at time zero and after a period of one hour, as the concentration increase of  $N_2O$  in the headspace of the chamber was found to be linear with time. In this project, the time of one hour was found, in preliminary measurements, to be almost the minimum necessary to accumulate enough gas in the chamber to give significant flux measurements, for the soils studied. The second kind of closed chamber used in the present research is an automated closed chamber (Figure 2.2, Smith *et al.*, unpublished).



**Figure 2.2** - Automated closed chamber system which includes the chamber and a box containing sampler, timer, pump and batteries.

The system is composed of two parts: 1) the chamber (70 x 70 cm in area, for 20 cm in height, 5 cm below ground and 15 cm headspace), made of galvanized steel and

provided with a lid, which can be closed mechanically by an electric actuator driven by a 24V motor, mounted on an extension frame attached to the chamber ; 2) a box containing a sampler (Figure 2.3), a timer, a pump, and batteries to power both the chamber lid mechanism and the sampler.



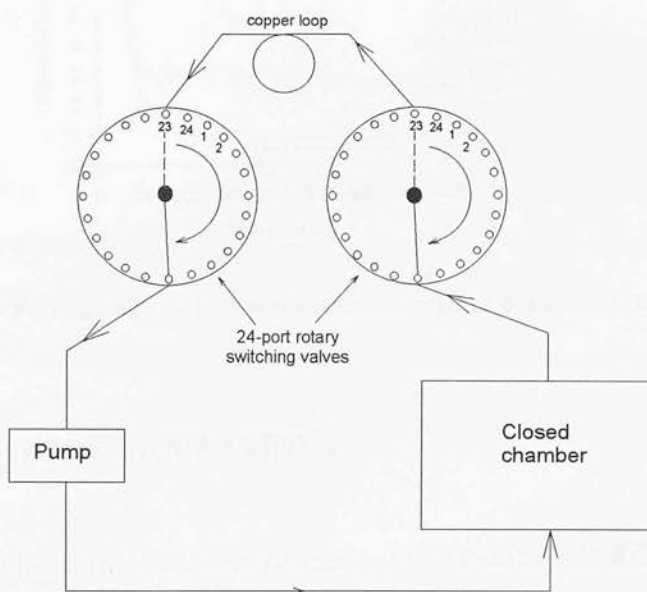
**Figure 2.3** - Sampler loop assembly of the automated chamber system. (1) 24-port valve, (2) copper tubing loops, (3) tubes with 3-way ports connecting the sampler to the pump and to the chamber.

The sampler consists of two 24-port rotary valves (Scani-Valve Co., S. Diego, USA) (1), activated by 24V motor drive, and fitted with 24 copper loops, each of 1 ml volume (2), where the gas samples are stored. Two plastic tubes (3), each closed by a 3-way valve, connect the rotary valve and the loops to the pump and the chamber, respectively (Fig.2.4).

A timer controls the closing and opening of the chamber lid, and activates the pump, which circulates air from the chamber through a loop, via the connection tube, then back into the chamber. The switching mechanism then moves the rotary valves to the



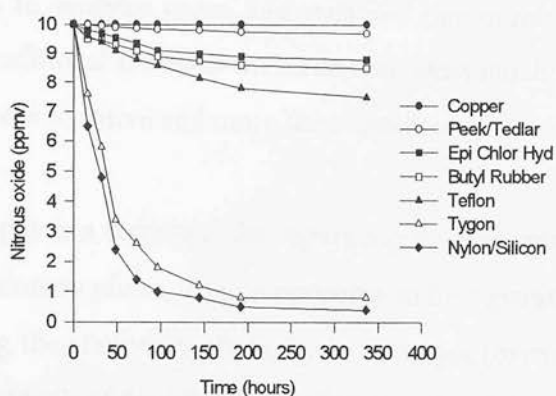
next position, isolating the filled loop, and connecting the next loop to the sampling system. This procedure is then repeated, at intervals preset with the timer. When all the loops are filled, the whole valve-loop assembly can be removed and brought back to the laboratory for automated gas chromatographic analysis.



**Figure 2.4** - Automated closed chamber and gas sampling system. Gas only circulates to flush loop at the end of the closure period. The two rotary valves are mounted one above the other, on the same 24V motor drive. Only one port/loop shown.

Another valve-loop assembly, in which each loop has been previously evacuated, can be installed to replace the filled one, and the flux measurements continued.

The loops are made of copper, with brass Swagelok fittings, in order to avoid loss of gas. A range of plastic and rubber polymers were tested, but all gave, except peak/tedlar, unacceptable losses of  $N_2O$ , when tested with standard gas mixtures (Scott and Crichton, unpublished data) (Figure 2.5).



**Figure 2.5** - Permeability of different materials to trace concentrations of  $N_2O$ .

## 2.3 LABORATORY INCUBATIONS

Laboratory incubation studies were carried out in 1 l resealable Kilner jars. The lids of the jars were provided with a hole fitted with a rubber grommet; a short tube (3 cm) was inserted in the hole and sealed to the grommet with superseal glue. A three-way stopcock was attached to the top of the tube, to sample gases in the jar. The jars were demonstrated to be adequately gas tight in a preliminary experiment. Gas samples were withdrawn with greased glass syringes and immediately analyzed by gas chromatography.

Soil incubated in the jars was either sieved at 2 mm or in the form of soil cores. Soil cores consisted of PVC cylinders 5 cm high and 5 cm in diameter. 4 cores could fit in a steel corer provided with a cutting tip. After the sampling, cores were removed from the corer, the bottom of each core was closed with a plastic lid to prevent loss of soil and each core was put in a jars. When the soil needed to be incubated in the presence of acetylene (see section 2.5 below), after closing the jars, a volume of air, equal to the volume of acetylene to be added, was withdrawn from the jars with a plastic syringe.

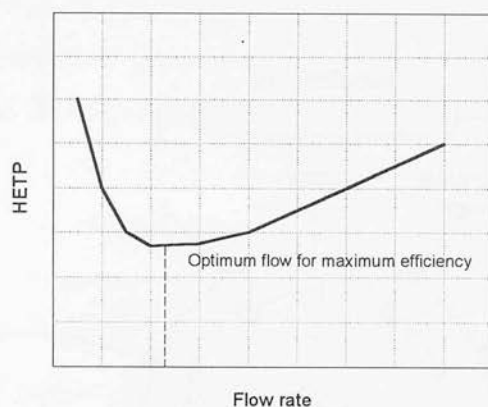
## 2.4 GAS CHROMATOGRAPHY

Since the end of 1950s, with the introduction of gas chromatographic methods, it has become possible to analyze gases and volatile compounds which were previously measured with traditional chemical methods that were much less specific for most of the compounds, less sensitive and more time consuming.

Gas chromatography is a technique for separating volatile substances by passing a gas stream over a stationary phase. The components to be separated are carried through a column containing the stationary phase, by an inert gas (carrier gas). If the stationary phase is solid we speak of Gas-Solid Chromatography; the column is packed with an adsorbent of small particle size, or the inner surface of a capillary column is coated with a thin adsorbent layer. If the stationary phase is an unvolatile liquid coated either on an inert support material or on the internal surface of a capillary column, then we speak of Gas-Liquid Chromatography.

The carrier gas must be inert, pure, suitable for the detector and preferably not too expensive. Commonly used gases are hydrogen, helium, nitrogen and argon. Column efficiency in separating the volatile compounds depends also upon choosing a suitable linear gas velocity. The optimum flow rate can be determined by making a Van Deemter plot of HETP (Height Equivalent to a Theoretical Plate) vs linear gas velocity (Figure 2.6). The most efficient flow-rate is at the minimum HETP (for more details see McNair and Bonelli, 1969). The simplest way to measure gas flow rate is with a soap-bubble flowmeter and a stopwatch.

Gases are introduced instantaneously into the column by gas-tight syringes, introducing the needle through a self-sealing septum (reproducibility around 2% relative), or via by-pass sample loops (reproducibility better than 0.5%). A better resolution is provided by automated GC injector systems (Smith and Harris, 1970; Parkin, 1985; Smith and Arah, 1991), which are also labour-saving, than by manual injection.

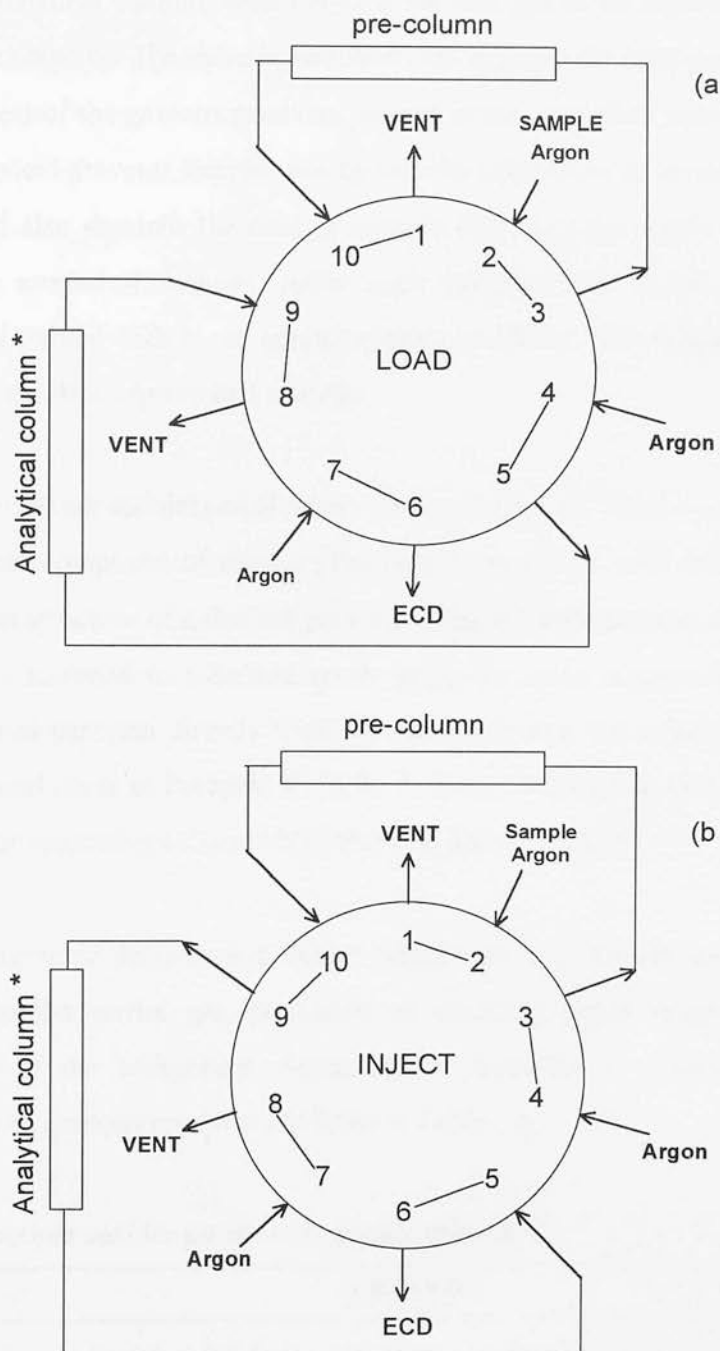


**Figure 2.6 - Van Deemter plot ( from McNair and Bonelli, 1969).**

The gas chromatographs used for the present research were equipped with an automated injection system based on the system described by Smith and Arah (1991) and Arah *et al.* (1994). In this system, an electrically actuated 10-port injection valve (Valco Instruments Company, Incorporated, Houston, Texas) is attached via three-way solenoid valves (Peter Paul Electronics Company, Incorporated, New Britain, Connecticut) to two electrically actuated 16-port rotary valves (Valco), equipped with 16 hypodermic needles as injection ports, to which syringes, vials or tedlar bags can be fitted.

The sampling loop is evacuated, isolated from the pump, and then coupled to the sample container, causing gas to flow into the loop. Following pressure equilibration, the contents of the loop are injected into the GC for analysis. The 10-port valve is connected to “pre-column” and “analytical” columns (Figure 2.7).

Immediately after the injection the 10-port valve is in the “LOAD” position, which allows the sample to be carried (by the carrier gas) through the pre-column. This system is mainly used to analyze  $N_2O$  with an ECD detector, and it allows the  $O_2$  in the sample to be separated in the pre-column from the  $N_2O$  peak, the former having a smaller retention time than the latter.



**Figure 2.7** - A scheme of the 10-port valve (a) in the LOAD position, (b) in the INJECT position. Argon is used as the carrier gas. \* Backflush after passage of  $N_2O$ .

After a predetermined time (23 sec for the system used in the present research), the 10-port valve is switched to the “INJECT position” and the sample is carried first through the analytical column, where  $N_2O$  is the first gas to be separated and passes directly to the detector. The valve is switched then again to the load position (after 60 sec) and the rest of the gaseous products present in the analytical column are flushed away. This system prevents interference by volatile substances of no interest with the  $N_2O$  peak and also shortens the time of analysis. For  $N_2O$  the whole cycle for each sample takes around 4 minutes. After each injection the system is repeatedly evacuated and purged with  $N_2$  to minimize memory effects. The whole system is PC controlled, as are data capture and analysis.

The columns used are stainless steel tubes, 6 mm in diameter, filled with Porapak Q, a porous polymer composed of ethylvinylbenzene cross-linked with divinylbenzene to form a uniform structure of a distinct pore size. The columns contain only the porous polymer beads screened to a defined mesh range, no liquid support is present. The solute molecules partition directly from the gas phase into the amorphous polymer. There are several kinds of Porapak, P, Q, R, S, T and N. Porapak Q is non polar and widely used for separating  $CO_2$  and  $N_2O$  from  $N_2$  and  $O_2$  samples.

The chromatographic detector is a device which measures the amount of separated components in the carrier gas, producing an electrical signal proportional to the concentration of the component. Some of the detectors in current use for the determination of gaseous products are listed in Table 2.2.

**Table 2.2** - Detectors used for gas chromatographic analysis.

<i>Detector</i>	<i>Application</i>
Thermal conductivity (TCD)	all gases (major constituents of mixtures)
Flame ionization (FID)	hydrocarbons (trace concentrations)
Helium ionization	all gases (trace concentrations)
Electron capture (ECD)	$N_2O$ ; $O_2$ ; $CO_2$ ; halogenated compounds
Ultrasonic	all gases (wide concentration range)
Flame photometric	sulfur and phosphorus compounds

In the present work a ECD, a TCD and an FID have been used to measure  $\text{N}_2\text{O}$ ,  $\text{CO}_2$  and  $\text{O}_2$ , and  $\text{CH}_4$  respectively.

In the ECD detector,  $\beta$ -radiation from a radioactive source (usually nickel-63) ionizes the carrier gas (nitrogen or argon or argon-methane), and an applied potential causes current to flow. Electrophilic compounds capture the electrons produced by the ionization and the current is reduced. This provides the basis for a highly sensitive and selective detector. The limit of detection for  $\text{N}_2\text{O}$  can be of the order of 1.0 ppbv  $\text{N}_2\text{O}$ .

The TCD detector employs a tungsten filament which is heated by passing a constant current through it. Carrier gas flows continuously over the heated filament and dissipates heat at a constant rate. When sample molecules mixed with the carrier gas pass over the hot filament, the rate of heat loss is reduced and the resistance of the filament increases. The resistance change is measured by a Wheatstone bridge and the signal fed to a recorder.

In the FID detector, hydrogen and air are used to produce a flame. A collector electrode with a DC potential applied is placed above the flame and its conductivity measured. As organic products are combusted, the low conductivity produced by the hydrogen increases and the current which flows can be amplified and fed to a recorder.

The gas chromatographs used for gas analysis were a Unicam 610 series equipped with a Pye Unicam PU4500 ECD detector, a Hewlett Packard HP 5890 II equipped with a TCD and a Unicam 610 series equipped with a Pye Unicam PU4500 FID detector. Details of the GC operative parameters for the analysis done in the present work are reported in Table 2.3.



**Table 2.3** - GC parameters as set up for the analysis in the present work.

	UNICAM 610 - ECD	HP 5890 II - TCD	UNICAM 610 - FID
Oven temperature	60 °C	65 °C	36-37 °C
Detector temperature	320 °C	80 °C	120 °C
Carrier flow rate	40 ml/min (Ar)	40 ml/min (He)	40 ml/min
Hydrogen flow rate	-	-	50 ml/min
Air flow rate	-	-	400 ml/min
Columns	1.5 m of 6mm Porapak Q	1.2m of 6mm Porapak Q for Air/CO <sub>2</sub> separation  1.5m of 3 mm Mol.Sieve 5A for O <sub>2</sub> /N <sub>2</sub> separation	1.5m of 6mm s.s.alumina
Column head pressure	6.5 psig	15 psig	-
Sample size	1 ml	0.5 ml	1 ml

## 2.5 THE ACETYLENE INHIBITION TECHNIQUE

The acetylene (C<sub>2</sub>H<sub>2</sub>) molecule has become of interest since it was discovered that it can be used to detect nitrogenase activity (Yoshinari *et al.*, 1977) denitrification activity (Klemedtsson *et al.*, 1977; Yoshinari *et al.*, 1977) and chemoautotrophic nitrification activity (Hynes and Knowles, 1982).

To quantify denitrification activity, 1-10 kPa of C<sub>2</sub>H<sub>2</sub> is introduced into the soil atmosphere. These very high concentrations have been found to be effective in inhibiting N<sub>2</sub>O reductase, the enzyme responsible for the reduction of N<sub>2</sub>O to N<sub>2</sub> (Balderstone *et al.*, 1976; Yoshinari and Knowles, 1976; Yoshinari *et al.*, 1977; Klemedtsson *et al.*, 1977). Since the reduction of N<sub>2</sub>O to N<sub>2</sub> is blocked, the N<sub>2</sub>O produced represents the total production of N<sub>2</sub> plus N<sub>2</sub>O. This makes it possible to

measure the  $N_2$  produced by denitrification, since the background atmospheric concentration of  $N_2O$  is low, unlike that of  $N_2$ .

Acetylene at very low concentrations (about 1-10 Pa  $C_2H_2$ ) has been found to inhibit the activity of ammonia monooxygenase in *Nitrosomonas europaea* (Hynes and Knowles, 1978). The  $C_2H_2$  acts as a "suicide substrate", inhibiting the enzyme, which catalyzes the oxidation of ammonia to hydroxylamine (Hynes and Knowles; 1982, Hyman and Wood, 1985). As *Nitrosomonas* is the most common nitrifier which can be found in soil, it is generally assumed that the inhibiting effect of low concentrations of  $C_2H_2$  on  $N_2O$  emissions is the result of  $C_2H_2$  inhibition on autotrophic nitrifying bacteria. Nitrifying activity led by heterotrophic microorganisms (bacteria, fungi, yeasts) is considered, instead, not sensitive to  $C_2H_2$ . Though this is widely accepted, experimental results to support this conclusion are very scarce. Of the most common scientific publications, which are used by authors as a reference to support the low sensitivity of heterotrophic nitrifiers to  $C_2H_2$ , only two works have been really carried out on an heterotrophic microorganism (see summary in Table 2.4) .

From Table 2.4 it is clear that some caution must be used when we want to distinguish autotrophic from heterotrophic nitrification activity by using  $C_2H_2$  because of the paucity of experiments on heterotrophic nitrifiers and because of the almost complete lack of information on the pathways of heterotrophic nitrification. Moreover, in the eventuality that heterotrophic nitrifiers would have an induced (opposed to a constitutive) nitrifying biochemical system, an in-vitro pure culture of heterotrophic microorganisms could lack of the "inducing/s" molecule/s , so that lack of nitrification could be the result of a wrong plating rather than  $C_2H_2$  inhibiting action.

In *Appendix I* a preliminary experiment is reported, carried out to test the minimum concentration of  $C_2H_2$  which was needed to inhibit  $N_2O$  autotrophic emissions in the two soils studied, where "autotrophic nitrification" is intended a nitrification pathway which involves the enzyme ammonia-monooxygenase. A value of 0.1% v/v (100 Pa)  $C_2H_2$  was found to be adequate.

**Table 2.4** - Most commonly reported references in studies where C<sub>2</sub>H<sub>2</sub> is used to distinguish between autotrophic and heterotrophic nitrification.

Authors	Journal	Inhibiting C <sub>2</sub> H <sub>2</sub> effect (+/-)	Organism or substrate tested
Hynes and Knowles, 1978	Fems Microbiol Lett 4:319	+	<i>N. europea</i>
Bremner and Blackmer, 1979	Nature 280: 380	+	Soil
Walter <i>et al.</i> , 1979	Soil Sci Soc A J 43:195	+	Soil
Mosier, 1980	Soil Biol Biochem 12:443	+	Soil
Berg <i>et al.</i> , 1982	Soil Biol Biochem 14: 301	+	Soil
Bleakley and Tiedje, 1982	Appl Envir Microbiol 44:1342	+	pure cultures*
Hynes and Knowles, 1982	Can J Microbiol 28: 334	+	<i>N. europea</i>
		-	<i>Arthrobacter</i> sp.
Hynes and Knowles, 1984	Can J Microbiol 30: 1397	+	<i>N. europea</i>
Schimel <i>et al.</i> , 1984	Appl Envir Microbiol 48: 802	+	<i>Aspergillus flavus</i> ACTT26214
Hyman and Wood, 1985	Biochem J 227: 719	+	<i>N. europea</i>
Martikainen, 1985	Appl Envir Microbiol 50:1519	+	Soil
Davidson <i>et al.</i> , 1986	Appl Envir Microbiol 52: 1280	+/-	Soil
Robertson and Tiedje, 1987	Soil Biol Biochem 14: 187	+/-	Soil
Klemetsson <i>et al.</i> , 1988a	Biol Fert Soil 6:106	+	Soil
Klemetsson <i>et al.</i> , 1988b	Biol Fert Soil 6:112	+	Soil
Bedard and Knowles, 1989	Review (see Bibliography)		
Klemetsson <i>et al.</i> , 1990	Review (see Bibliography)		

\* nitrification inhibited but N<sub>2</sub>O produced anyway. Fungi did not produce N<sub>2</sub>O in absence of NH<sub>4</sub><sup>+</sup>.

## 2.6 USE OF ANTIBIOTICS TO DISTINGUISH BETWEEN FUNGAL AND BACTERIAL ACTIVITY IN SOIL

### 2.6.1 BACKGROUND

The study of the action of antibiotics began around 1940, when penicillin became available (Gardner, 1940, Duguid, 1945) and when Woods and Fildes (1940) enunciated the principle of inhibition of essential metabolism by structural analogues. Since then, the studies on the mode of action of this compounds have expanded at

different levels of investigation: effects on cell metabolism, permeability and integrity of the cell membrane, interference with proteins and nucleic acid synthesis, etc.

Antibiotics are organic substances produced by microorganisms themselves, which are either toxic or growth-inhibitory for other organisms. Particularly useful, when a physiological distinction is required between prokaryotic and eukaryotic cells, are the antibiotics which block protein synthesis (Table 2.5). While some antibiotics (e.g. puromycin) are capable of inhibiting protein synthesis in cell-free systems from any source, others show selective toxicity determined by the type of ribosome involved. The most widely used for distinguishing between bacterial and fungal activity in soil are streptomycin and cycloheximide.

**Table 2.5** - Classes of antibiotics which act on targets specific to either prokaryotic or eukaryotic cells (from. Strainer *et al.*, 1976).

ANTIBIOTICS	MODE OF ACTION	ACTIVE AGAINST	
		PROKARYOTES	EUKARYOTES
Penicillins	Block synthesis of peptidoglycan constituent of cell wall	+ <sup>a</sup>	-
Polyene antibiotics	Combine with sterols in cell membrane; affect permeability	_b	+
Glutarimides	Block synthesis of proteins on 80S ribosomes	-	+
Aminoglycosides, tetracyclines, macrolides, chloramphenicol	Block synthesis of proteins on 70S ribosomes	+	_c

<sup>a</sup> Except those who not produce cell walls; <sup>b</sup> Some mycoplasmas that incorporate sterols from the growth medium into the membrane are sensitive; <sup>c</sup> At high concentrations they may affect organellar protein synthesis.

### 2.6.2 THE TARGET

The biochemical activities of a cell can be stopped either by damage to existing enzymes and structures or by inhibition of the formation of the new ones needed for cell growth and division. The activities of cells depend upon enzymes; each enzyme is a specific protein whose properties are determined by its three-dimensional structure and this, in turn, is determined by its primary sequence of amino acids. The setting up of the primary sequence in the course of protein synthesis is a key process. The

information that determines the primary sequence of a protein is encoded in the DNA of a cell; the information held in the DNA is first *transcribed* into messenger RNA, which binds to the smaller ribosomal sub-unit of the complete ribosome and is there *translated* into a specific sequence of amino acids, peptide-bonded by the action of the ribosome. Bacterial ribosomes, with sedimentation coefficients of 70S, consist of two unequal subunits of 50S and 30S. Eukaryotic ribosomes, sedimentation coefficient 80S, consist of two sub-units 60S and 40S. The ribosome itself is a complex of proteins and RNA: the 50S subunits of the bacterial ribosome contain some 32, and the subunit 30S some 21, different proteins (Stryer, 1988).

Selective interference is certainly possible: prokaryotic and eukaryotic ribosomes differ in sedimentation coefficient, protein content, and their ability to bind antibiotics such as chloramphenicol, cycloheximide and streptomycin (Gale *et al.*, 1981).

### 2.6.3 STREPTOMYCIN AND CYCLOHEXIMIDE: INHIBITORS OF THE SMALLER RIBOSOMAL SUBUNIT

**Streptomycin:** when this aminoglycoside (Figure 2.8) is added to cultures of sensitive cells several effects are observed: inhibition of protein synthesis (Fitzgerald

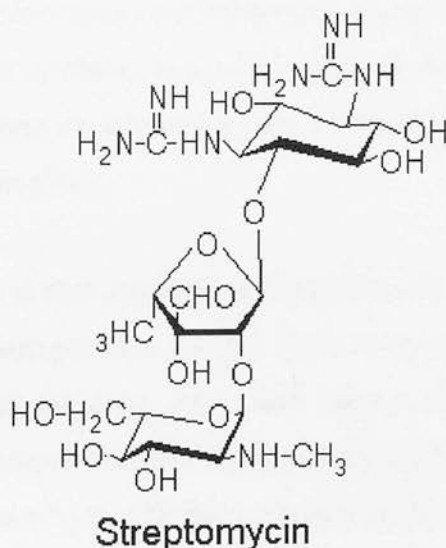


Figure 2.8 - Streptomycin molecule.

*et al.*, 1948); stimulation of RNA synthesis (Stern *et al.*, 1966); inhibition of cellular respiration and membrane damage (Dubin *et al.*, 1963) and , ultimately, cell death.

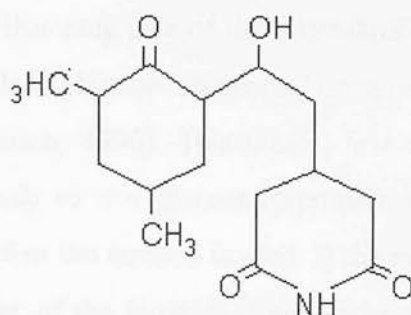
The main effect has been demonstrated to be the inhibition of prokaryotic protein synthesis, while the other effects seem to be secondary consequences.

The streptomycin binds the subunit 30S of the prokaryotic ribosome, interacting with the ribosomal protein S12 (formerly P10). This protein controls the overall efficiency of the mRNA translation at the level of binding of tRNA to the ribosome. The effect of such an interaction is complex. it seems that streptomycin can inhibit aspects of all phases of protein synthesis (initiation, elongation, termination) in addition to enhancing ambiguity of translation of mRNA.

**Cycloheximide**: it belongs to the glutarimide antibiotics, most of them characterised by having a  $\beta$ (2-hydroxyethyl)glutarimide moiety attached to the cyclic ketone (Figure 2.9). Glutarimide antibiotics do not inhibit prokaryotic systems, but a wide range of eukaryotic cells (e.g. yeasts, fungi, algae, *Tetrahymena*, mammalian cells and tumor cells in culture) are sensitive to cycloheximide (Sisler and Siegel, 1967; Obrig *et al.*, 1971). Kerridge (1958) showed that cycloheximide was a potent inhibitor of protein synthesis in cells of *Saccharomyces carlsbergensis*. More recently the drug has been shown to inhibit protein synthesis in a wide range of eukaryotic cells but not in prokaryotic ones. Moreover, cycloheximide was not found to inhibit protein synthesis in mitochondria and chloroplasts.

The general observation is that cycloheximide prevents the movement of ribosomes along mRNA. There is also evidence that the chain initiation could be more sensitive than chain elongation to the drug. Ribosomal subunit 60S was identified as the determinant of the response to the drug. Cycloheximide has also been shown to have a potent effect on DNA synthesis. The exact mechanism of this inhibition and how this is related to protein blockage of synthesis is still unclear.





**Cycloheximide**

**Figure 2.9 - Cycloheximide molecule.**

#### 2.6.4 THE ANTIBIOTIC BLOCK TECHNIQUE AS A WAY OF DISTINGUISHING BACTERIAL FROM FUNGAL ACTIVITY IN SOIL

The selective inhibition technique has been used to differentiate the activity of fungi and bacteria in soil (Anderson and Domsch, 1973, 1975; West, 1986).

Streptomycin and cycloheximide are the most commonly used antibiotics as they have been demonstrated to be the most effective when prokaryotic and eukaryotic microorganisms are in active growth, following addition of readily-utilizable C substrates (Anderson and Domsch, 1973). Indeed, the principle, on which the technique was developed and is based, is that streptomycin and cycloheximide, being inhibitors of protein synthesis, can have an inhibiting action only on organisms which are undergoing instant proliferation or anyway are synthesising enzymes *ex novo* (Wardle and Parkinson, 1990).

A substrate is used to induce active protein synthesis, which is added in a non-limiting quantity for the period of observation. In the classical method by Anderson and Domsch (1973), glucose was chosen as an ideal substrate to induce an active growth and respiration, as it was considered that both bacteria and fungi were able to use it as a source of energy and growth. However, less than half of the total microbial biomass in soil has been estimated to be active (Ingham and Klein, 1982, 1984; Ingham and

Horton, 1987), so that only part of the microflora will promptly respond to glucose addition, the so-called “glucose-responsive” component of the total microbial biomass (Wardle and Parkinson, 1990). This means, first of all, that the inhibition method probably applies only to this glucose-responsive component and two assumptions need to be made when the method is used: i) the ratio *bacteria:fungi* in the inhibitor sensitive component of the biomass is the same as that in the inhibitor-insensitive component (West, 1986); ii) that bacterial and fungal components of the microbial biomass respond equivalently to glucose (Wardle and Parkinson, 1990). However, Stamatiadis *et al.* (1990) reported that the fungal:bacterial biomass ratio declined greatly after glucose amendment in two soils during the first 24 h of incubation, illustrating a selective advantage of bacteria over fungi in assimilation of a readily utilizable substrate such as glucose. These findings, however, cannot be generalised.

Particularly important is the length of the incubation with antibiotics. Anderson and Domsch (1973), in outlining the method, suggested that the experiment should be kept as short as possible in order to avoid confusing initial population ratios with those derived from the successive populations which would develop in the treated soil. Periods between a few hours and 48 hours have been considered acceptable, depending on the soil, while it has been generally found that for long incubation periods, between 3 and 10 days, the effectiveness of the inhibition is lost and results become quite complicated to interpret (Anderson and Domsch, 1973; Stamatiadis *et al.*, 1990; Landi *et al.*, 1993). One of the reasons is that, in long-term incubations, populations of microorganisms resistant to the antibiotics could develop, utilising the biocide-killed cells as substrate for growth and respiration, so that even a positive flush of CO<sub>2</sub> could be observed where antibiotics have been added, as compared with a control.

Moreover, care should be taken to keep the concentration of antibiotic at a level which is low enough not to have non-target effects on other components of the soil microflora. This last condition has been shown to be quite problematic (Anderson and Domsch, 1973; Ingham and Coleman, 1984; Landi *et al.*, 1993), as the real effect of



different concentrations of antibiotics on the microbial biomass and activity is quite complicated, the biocides being at the same time both substrates for and inhibitors of soil mineralization (Stamatiadis *et al.*, 1990, Landi *et al.*, 1993; Badalucco *et al.*, 1994).

If the correct assumptions are made and the determination of the *fungus:bacterial* ratio is explicitly referred to the sole microbial component stimulated by the substrate addition, then the method provides an interesting tool for the distinction of fungal versus bacterial activity. In fact, other methods, such as the direct count of number of microorganisms (Witkamp, 1963), or indirect calculations by extrapolation from metabolic indexes derived from pure cultures (Satchell, 1971), or statistical approaches by comparison of correlation coefficients (Witkamp, 1973), or analyses for the presence and quantity of products associated with unique microorganisms (Swift, 1973), etc., have been demonstrated to be valid only in special cases.

## **2.7 LABORATORY MEASUREMENTS OF SOIL PHYSICO-CHEMICAL PARAMETERS**

### **2.7.1 EXTRACTABLE $\text{NH}_4^+$ -N AND $\text{NO}_3^-$ -N**

The quantity of nitrogen present in soil as extractable mineral-N, a term generally applied to ammonium ( $\text{NH}_4^+$ ), nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ), is quite small as compared with organic-N or the N present in minerals. Though this pool of nitrogen is quite small, it is very important as it represents a very active pool, which contains the N forms which plants and microbes utilize. Not being covalently bound to the organic matter, or blocked in the rocks, these forms of N can be extracted from the soil with potassium chloride or sulphate solutions.

Soil is generally extracted immediately after sampling, or alternatively is stored at low temperature. Extracting solution and soil (5:1 ratio) are shaken for 1 h and then filtered through a Whatman 42 filter paper. The extracting solution used in this work

was 1 M KCl. The molarity of the solution used generally depends on the quantity of mineral N present in the soil, the cation exchange capacity of the soil and the soil composition. The extracting solution will remove soluble and exchangeable ammonium, nitrite and nitrate which can then be measured separately or together. KCl extracts can be stored for up to two months at 2 °C before analysis, though a maximum of 2 weeks is advisable to avoid slight changes in ammonium concentration.

For the measurements of ammonium-N and nitrate-N an automatic colorimetric method was used, which can be performed by an AutoAnalyzer equipped with a colorimeter with a flow cell. The method is based on a series of subsequent reactions which, starting from ammonium or nitrate, produce coloured products which are quantified colorimetrically.

$\text{NH}_4^+$ -N was determined with a modified version (Chemlab Instruments Ltd) of the method by Crooke and Simpson (1971), which is based on the Berthollet reaction. In the classic method alkaline phenol and hypochlorite react with ammonia to form a blue indophenol. As both reagents are unstable and toxic, salicylate (2-hydroxybenzoate) is substituted for the former and dichloroisocyanurate, which decomposes in alkaline solution to form hypochlorite ions, for the latter. Nitroprusside is added as a catalyst. Citrate buffer is used to reduce any slight acidity. The absorbance of the emerald-green colour which develops is measured at 650 nm.

$\text{NO}_3^-$ -N was determined with a modified version (Chemlab Instruments Ltd) of the method by Best (1976). The method is based on the reduction of nitrate to nitrite by hydrazine-copper reagent (a mixture of hydrazine sulphate and cupric sulphate in water) and determination of the total nitrite content by the formation of an azo dye, with sulphanilamide and naphthylethylenediamine. The absorbance of the highly coloured azo dye is measured at 520 nm. To determine the nitrite concentration alone deionized water is used instead of hydrazine-copper reagent. In this case only the nitrite already present gives a colorimetric reaction; nitrate can be then measured by difference.

### 2.7.2 ORGANIC CARBON

Organic C in soil was determined by the titrimetric method of Kalembasa and Jenkinson (1973). With this method soil is previously reduced to powder in order to facilitate the digestion. 0.1 - 0.2 g of soil are weighed, depending on the C content of the soil. Soil organic C is completely oxidized by gently boiling the soil for two hours with an acid dichromate solution, in digestion flasks fitted with a Liebig condenser, at 130-135 °C on an electric hot plate. After cooling, the condenser is rinsed with distilled water (100 ml) and a few drops of indicator (barium diphenylamine p-sulphonate) are added. The excess dichromate is then titrated with ferrous ammonium sulphate (0.2 N). The colour change is from dark violet to emerald-green. The amount of dichromate consumed is that left in the blank digestion without soil less that remaining in the soil digestion. In the overall reaction one mole of C reacts with 3 moles of  $K_2Cr_2O$  from which the C content can be calculated.

### 2.7.3 TOTAL CARBON AND NITROGEN

Total carbon and nitrogen in the soil were determined by mass spectrometry using a mass spectrometer VG Micromass 622 interfaced with a Carlo Erba 1400 automatic Nitrogen Analyzer via capillary tubes connected to a three-way valve, which lets 2% of the gas stream from the N analyzer flow into the mass spectrometer.

When total carbon was measured the column of Carbosorb, which normally absorbs  $CO_2$  from the gas stream in the automatic analyzer, was substituted with a column of glass beads.

Soil was previously air-dried and reduced in fine powder and samples of 5-10 mg were used for the analysis.  $(NH_4)_2SO_4$  (21% N) and acetanilide (10.36% N) were used as standards for total N, while sodium-oxalate (17.5% C) and acetanilide (71.6% C) were as standard for total C.



#### 2.7.4 SOIL pH

10 g of air-dried soil, sieved with a 2 mm mesh sieve, are shaken with 25 ml of deionized water for half an hour on a shaking machine. The solution is then stirred, and the pH is measured inserting a glass electrode in the solution; pH is recorded after about 30 seconds. The pH meter is calibrated before the measurement with buffer solution at pH 4.0 and 7.0. Calibration for routine purposes is generally carried out at ambient temperature.

#### 2.7.5 SOIL WATER CONTENT

Water content of soil is generally expressed as “gravimetric water content ( $\theta_g$ )”, i.e., the mass of water per unit mass of oven-dry soil, or as “volumetric water content ( $\theta_v$ )”, i.e. the volume of water per unit volume of soil, typically  $\text{cm}^3 \text{H}_2\text{O cm}^{-3}$ . For routine purposes the chosen method was the former. About 10 g of fresh soil were weighed in small cups and placed in an oven at 105 °C overnight. The next morning samples were cooled in a desiccator and reweighed. Typically results are expressed as  $\text{g g}^{-1}$  or as a percentage.

#### 2.7.6 SOIL BULK DENSITY AND WATER FILLED PORE SPACE

Soil bulk density represents the mass of oven-dry soil present in a given volume of naturally structured soil. For the measurement, a metal cylinder is placed against the soil and gently hammered into the soil. It is then excavated, the soil surfaces smoothed flush with the ends of the cylinder, using a knife, and the ends are closed with plastic lids. In the laboratory, the cylinder is then sealed in a polythene bag. The cylinder is placed in the oven at 105 °C till the weight stabilizes. The volume and the weight of the empty cylinder is required, as well as the fresh and the dry weight of the bulk soil sample. The bulk density is then given by:



$$\text{Soil bulk density} = \text{mass dry soil} / \text{bulk volume of soil}$$

This method becomes problematic when a lot of roots or stones are present, or when soil has a high clay content and is quite dry, so that cracks can be present at the soil surface, causing difficulties in choosing a representative sample. In our case, the studied soil did not present any of these problems.

The water-filled pore space (WFPS), often expressed as a percentage, is the ratio of volumetric soil water content ( $\theta_v$ ) to total porosity of the soil ( $\varepsilon$ ), i.e.  $[100 \times \theta_v] / \varepsilon$ , where  $\varepsilon = \text{cm}^3 \text{ pore space} / \text{cm}^3 \text{ soil}$ . The volumetric water content can be easily derived from the gravimetric water content as it is equal to:

$$\theta_v = \theta_g \times \text{bulk density} / \text{density of water}$$

The total porosity of soil can be deduced from the following relationship:

$$\varepsilon = 1 - [\text{bulk density} / \text{particle density}]$$

Some typical values of particle density and porosity of soil are reported in Table 2.6.

**Table 2.6** - Reproduced from D. L. Rowell (1993).

	<i>Particle density</i> ( $\text{g cm}^{-3}$ )	<i>Bulk density</i> ( $\text{g cm}^{-3}$ )	<i>Porosity</i> ( $\text{cm}^3 \text{ cm}^{-3}$ )
Cultivated mineral soils, plough horizons	2.60	0.8 - 1.4	0.69- 0.46
medium-heavy textured, light texture	2.60	1.1 - 1.7	0.46 - 0.35
Subsoils and parent materials	2.65	1.2 - 1.8	0.47 - 0.32
Grassland and woodland, A horizons	2.4	0.8 - 1.2	0.48 - 0.50
Peats	1.4	0.1 - 0.3	0.93 - 0.79

Although  $\theta_v$  and  $\theta_g$  parameters cannot be easily compared among soils with different textures, the WFPS can be used, as it takes into account the total porosity of the soil and the compaction, and is consequently directly related to gas diffusivity.

#### 2.7.7 WATER RELEASE CURVE

Water retention in soil depends on forces acting between water molecules and hydrophilic particle surfaces present in and between the soil pores. This complex of forces can be considered as a form of potential energy and is generally expressed as units of pressure. If the pressure of the soil water is less than the pressure of pure free water, if a drop of water is added to moist soil, it will be absorbed by the soil, as a result of the pressure difference. The amount by which the pressure is less than atmospheric pressure is termed the “soil water suction” or “soil water tension”.

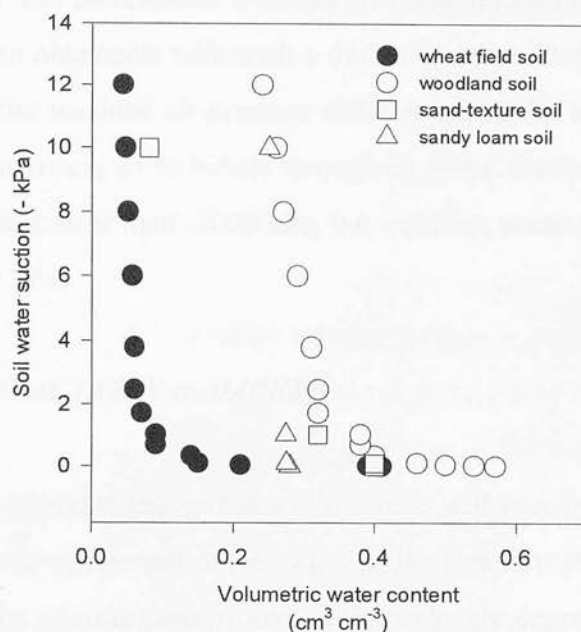
The water tension or matrix potential in the soil can vary between 0 Pa, at the saturation water content, to  $-10^3$  MPa when soil is completely air-dried.

At the field capacity (only micropores are water filled) the soil water tension is around  $-3.3 \times 10^3$  Pa.

The relationship between soil water content and soil water suction is known as the *water release curve* or *water release characteristic*. The shape of the curve will depend on the pore size distribution and so on the soil texture.

In Figure 2.10 water release curves for the two soils studied (the wheat field soil and the woodland soil) are compared with values obtained from a sandy soil and a sandy loam soil taken from literature. It can be seen that the wheat field (loamy sand) and the sandy soil, which are very loose and light soils, have lost most of their water when they have drained at field capacity. The woodland soil (sandy loam) and the sandy loam soil instead show a higher capacity to retain water.

In order to construct a water release curve, two methods have been used: the tension plate or table and the pressure chamber.



**Figure 2.10** - Water release curve for the wheat field soil and the woodland soil studied and for a sandy-textured soil (Hoogmoed and Klaij, 1990) and a sandy loam soil (Rowse, 1975).

The tension plate or table is used to measure the water release characteristic between saturation and ca -80 kPa. Small metal rings (4 cm diam.), provided with a fine mesh net at the bottom, containing the soil, are first brought to saturation and then placed on a very fine sand bed set on a water table, all covered to avoid evaporation. The plate is connected to a system of capillaries provided with a funnel or a graduated tube. By lowering the funnel a suction can be applied to the soil water which is drawn through the sintered plate until equilibrium is reached. Soil water suction is measured by the equivalent hydraulic head, and changes in soil water content by changes in the weight of the samples.

Matrix suction values considerably greater than -100 kPa (-2000 kPa or even more) can be obtained by increasing the pressure of the air phase in a pressure chamber or pressure membrane apparatus. A chamber is built so that a pressure above atmospheric can be imposed on the samples (the small soil-filled cylinders). The soil

water is connected to the exterior of the pressure cell via a porous ceramic plate or membrane. Water will be removed from the soil until equilibrium is established. The limit of the suction obtainable with such a device is determined by the design of the chamber and by the maximal air-pressure difference that the saturated porous plate can bear without allowing air to bubble through its pores. Ceramic plates generally do not hold pressures greater than -2000 kPa, but cellulose acetate membranes can hold more than 10,000 kPa.

#### 2.7.8 SOIL EXTRACTABLE $\alpha$ -AMINO-N

The amino acids, peptides and proteins available in soil in a free form are generally only a minor organic component of the soil, as in the free form they represent a source of N and C for the microorganisms and are immediately degraded. This component can be extracted by shaking the soil within an aqueous solution and can be measured with methods based on the ninhydrin reaction.

When an aqueous solution of an  $\alpha$ -amino acid is treated with ninhydrin (triketohydrindene hydrate), a violet colour is produced. In the first stage of the reaction, the amino acid is oxidized to give an  $\alpha$ -imino acid. This is further hydrolyzed to an  $\alpha$ -keto acid and ammonia. The ammonia reacts further to give the violet pigment and the  $\alpha$ -keto acid decarboxylates to give an aldehyde. The violet solution shows a significant absorption at 570 nm, and the intensity of absorption is proportional to the  $\alpha$ -amino acid present. This specific reaction does not occur with proline as it is a secondary amino acid; the product obtained in this case is different and absorbs at another wavelength. Alternatively  $\text{CO}_2$  or  $\text{NH}_3$  formed in the course of the reactions can be measured.

In the colorimetric method 2 ml of each extract were placed in a test tube, to which 1 ml of ninhydrin reagent (Sigma Chemical Co.) (Moore and Stein, 1954) was added slowly, mixing thoroughly. Tubes were then placed in boiling water for 25 minutes and subsequently cooled down at room temperature. 20 ml of ethanol-water (1:1)

were added and the absorbance of the solution was measured with a spectrophotometer at 570 nm wavelength. The concentration in solution was calculated from a calibration curve prepared with the same procedure, using L-leucine as a standard.

### 2.7.9 SOIL MICROBIAL BIOMASS

Because of the fundamental role which microorganisms have in soil nutrient cycling processes, considerable attention has been given in recent years to the development of methods for measuring soil microbial biomass (Jenkinson, 1988) and to the refinement of such methods in order to achieve a greater agreement between the results obtained with different techniques (Jenkinson, 1988; Sparling and Zhu, 1993). Among the available methods to estimate microbial C and N there are the fumigation-incubation method (FI) for microbial C (Jenkinson and Powlson, 1976) and N (Shen *et al.*, 1984), the fumigation-extraction method (FE) for microbial C (Vance *et al.*, 1987; Sparling and West, 1988) and N (Brooks *et al.*, 1985), the substrate-induced respiration method (SIR) (Anderson and Domsch, 1978) and the ninhydrin-positive compounds (NPC) extracted after fumigation (Amato and Ladd, 1988; Joergensen and Brookes, 1990). All methods offer some advantages and some disadvantages.

The FI technique is not reliable with acid soils ( $\text{pH} < 5$ ) (Vance *et al.*, 1987) or with soil amended with fresh organic C (Ocio and Brooks, 1990). The SIR technique requires to be calibrated against the other techniques before biomass estimation and in static systems neutral and alkaline soils can give erroneous results (Martens, 1987; Sparling and West, 1990). The FE and NPC methods are more reliable on acidic or freshly amended soils (Vance *et al.*, 1987; Ocio and Brooks, 1990) but present the problem of finding the appropriate factors to convert extractable C and N, from the flush, to microbial biomass C and N (Sparling and Zhu, 1993).

In the experimental work of this thesis the microbial biomass was measured by the ninhydrin-positive compounds method (NPC). The soil was first fumigated: 10 of

each sample were placed in a vacuum desiccator with a beaker containing 50 ml of purified chloroform, the desiccator was evacuated and the soil was left for 24 h in the chloroform atmosphere. After 24 h chloroform was removed and the soil was extracted with 1M KCl (see § 2.7.1.) On each extract the ninhydrin-positive compounds were measured (see § 2.7.8).

The ninhydrin-N deriving from the biomass is calculated as the ninhydrin-N of the fumigated samples minus the ninhydrin-N of the unfumigated samples. From the value obtained it is possible to calculate biomass C, N and dry matter as follows (Ocio and Brookes, 1990):

$$biomass-C = 31 \times \text{ninhydrin-N}$$

$$biomass-N = 4.6 \times \text{ninhydrin-N}$$

$$biomass \text{ (dry matter)} = 62 \times \text{ninhydrin-N}$$

There are big discrepancies in the  $K_{EN}$  factor used to calculate biomass value, because of the difficulty of obtaining an absolute measure of biomass to allow a calibration of the method (Jenkinson, 1988; Schimel *et al.*, 1989; Tate *et al.*, 1993; Greenfield, 1995). For this reason the NPC method is usually more reliable as a relative than as an absolute measure of microbial biomass (Tate *et al.*, 1988).

On this basis, the microbial biomass has been expressed as "*microbial biomass ninhydrin-N*":

$$"biomass \text{ ninhydrin-N}" = (\text{ninhydrin-N})_{\text{fumigated}} - (\text{ninhydrin-N})_{\text{unfumigated}}$$

#### 2.7.10 STATISTICAL ANALYSIS

All the statistical analyses have been performed using Sigmastat 1.0 (Jandel, Corporation).



To test which independent variable contributed significantly to predicting the dependent variable, a forward stepwise linear regression was performed. A multilinear regression was then used to find the model containing the independent variable which gave a significant contribution. To compare sample populations for significant differences a t-test was performed.

The influence of the different treatments on the samples, or of different incubation times on the same treatment, was determined using a one way RM analysis of variance. An "all pairwise" comparison was performed by the Student-Newman-Keuls test. Where necessary, data were logarithmically transformed to normalise them before the test was performed. Where not otherwise stated, significant difference between samples was set at  $P < 0.05$ .

Where not otherwise stated the bars in the graphs represent one standard error of the mean.

## FIELD EXPERIMENTS

### 3.1 INTRODUCTION

In the middle latitudes the major contribution to global  $\text{N}_2\text{O}$  emissions comes from agricultural lands subjected to fertiliser N inputs; however, an important contribution also comes from temperate forests, which often receive high concentrations of N by dry or wet deposition (see chapter 1, § 1.3).

The present field experiments, carried out in the woodland and in the wheat field (§ 2.1), aimed:

1. to measure  $\text{N}_2\text{O}$  fluxes from soil and the main environmental parameters, such as soil  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , soil water content, soil and air temperature, precipitation, in order to evidence: i) the main environmental factors which influence  $\text{N}_2\text{O}$  fluxes in the studied light textured soils; ii) the kind of relationships which relate those parameters to  $\text{N}_2\text{O}$  fluxes; iii) which kind of biological process (oxidative, reductive) might be more relevant for  $\text{N}_2\text{O}$  production in those soils;
2. to evaluate the effect of increased atmospheric N inputs on  $\text{N}_2\text{O}$  fluxes in the woodland, and at the same time the importance in the frequency of sampling by using different chamber systems (automated versus manual);
3. to evaluate at a smaller scale and in more detail, the relationship between  $\text{N}_2\text{O}$  fluxes, soil parameters and microbial biomass and activity, by the use of a core technique.

## 3.2 EXPERIMENTAL DESIGN

### 3.2.1 SEASONAL $N_2O$ FLUXES IN THE FIELD AND THEIR RELATION WITH ENVIRONMENTAL PARAMETERS.

Seasonal fluctuations of  $N_2O$  fluxes were followed in the wheat field and in the woodland described in chapter 2 (§ 2.1). Four static manual chambers (§ 2.2.2) were placed in each site in May 1994 and were not removed till the end of the experiment in May 1995, except when agricultural practices (ploughing, sowing, cropping) required their removal. The number of chambers which were used was probably less than optimal to measure  $N_2O$  fluxes from the field, as  $N_2O$  fluxes are reported to be extremely variable due to soil heterogeneity (Ambus and Christensen, 1994). However, it was not possible to increase the number of chambers in the wheat field because of the damage caused to the crop by walking through it to take the samples, as the field was part of a commercial farm. The same number of manual chambers was used into the woodland in order to have comparable results with the wheat field. In Table 3.1 are reported the rates of N fertilizer applied to the wheat field in the form of  $NH_4NO_3$ .

**Table 3.1** - Rates of fertiliser-N applied to the wheat field in Gullane from spring 1993 to spring 1995.

YEAR	PERIOD OF THE YEAR	RATE OF $(NH_4NO_3)$ -N APPLIED
1993	Spring	150 kg N ha <sup>-1</sup>
1994	(1) End of February/ Beginning of March	220 kg N ha <sup>-1</sup>
1995	(2) End of February/ Beginning of March	70 kg N ha <sup>-1</sup>
“	(3) 2 <sup>nd</sup> week of April	150 kg N ha <sup>-1</sup>

The wheat was usually sown in October-November, and started to flower in spring and the mature crop was harvested at the end of August - beginning of September.

At each sampling event, gas samples were collected from the chambers with 5 ml greased-glass syringes and analysed by electron capture gas chromatography (see § 2.4); soil was sampled from the top 10 cm (five randomly taken subsamples) and extracted the same day with KCl (§ 2.7.1). Water content was measured as well on the same day. KCl extracts were stored at 4 °C and later analysed for  $\text{NH}_4^+$  and  $\text{NO}_3^-$  (§ 2.7.1).

A temperature probe was inserted in the soil to 10 cm depth at both sites to record the soil temperature daily. A weather station in Gullane recorded precipitation daily, while air temperature information was obtained from the nearest available weather station in Dunbar, a few km from Gullane.

### *3.2.2 N<sub>2</sub>O FLUXES AND MINERAL N TRANSFORMATIONS IN SOIL FOLLOWING (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> FERTILISATION OF THE WOODLAND SOIL.*

On 11<sup>th</sup> of March 1995, two plots of 12 m<sup>2</sup> each (3 m x 4 m) were established in the woodland site. Both plots were located between the trees, and contained only a few nettle plants and a very sparse litter layer. One plot was fertilised with 20 kg N ha<sup>-1</sup> as ammonium sulphate, which was sprayed on the soil as a solution in distilled water (~ ½ l of solution m<sup>-2</sup>). This quantity of fertilizer was chosen in order to add a significant quantity of N without exceeding too much the quantity of N that a natural environment such as the woodland could experience in the temperate regions (~ 20 kg N ha<sup>-1</sup> y<sup>-1</sup>). The form of N fertilizer was chosen because preliminary laboratory experiments had already shown that N<sub>2</sub>O emissions from the woodland soil were more enhanced by addition of ammonium than by addition of nitrate. Moreover, among the reduced forms of N fertilizer, ammonium is generally reported as the most common form of N which arrive to the soil by atmospheric depositions.

The second plot was sprayed with distilled water (same quantity as for the treated plot) and was designated as control. Before the spraying the litter was removed in order to optimise the distribution of the N solution on the soil surface.

In each plot, an automated closed chamber and two manual closed chambers (§ 2.2.2) were inserted in the ground, after spraying, for the measurement of  $\text{N}_2\text{O}$  fluxes.

A temperature probe was buried in the space between the two plots, and was set to record the temperature at the ground surface, and at 5 cm and 10 cm depth, every hour.

At each sampling date, starting from the event of fertilisation, 4 random soil samples were taken at 0-10 cm, 10-20 cm and 20-30 cm, from each plot, and were analysed the same day for  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and water content (§ 2.7.1. and § 2.7.5).

### *3.2.3 APPLICATION OF A SOIL CORE METHOD TO INVESTIGATE THE RELATIONSHIP BETWEEN $\text{N}_2\text{O}$ FLUXES AND SOIL PARAMETERS.*

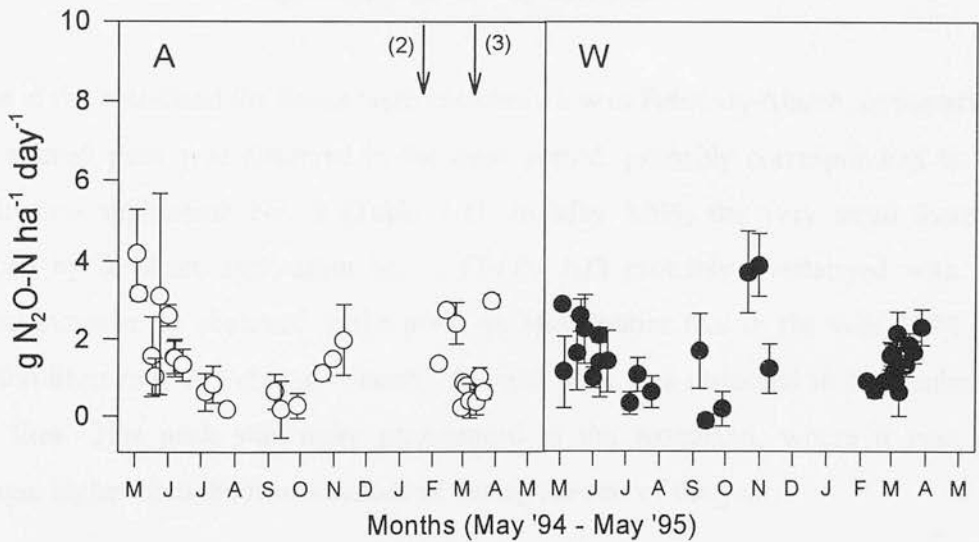
Soil cores were sampled in winter '94 -'95. A sampling period was chosen when the peak of mineral N in the arable soil had disappeared, in order to be able to compare the background  $\text{N}_2\text{O}$  fluxes in both soils. Soil cores (§ 2.3) were collected with a steel corer in the woodland and in the wheat field site from the top 20 cm of soil. The corer contained a cylindrical liner in four 5 cm sections (5 cm of diameter). Immediately after the sampling the corer was opened, the core divided into four sections with a knife and each section was capped on both extremities. Loose soil was sampled as well at 0-5 cm, 5-10 cm, 10-15 cm and 15-20 cm depth. The samples were brought back to the laboratory and the soil was analysed the same day for mineral N and water content. The next day, the cores and 100 g of loose soil were each incubated in 11 gas-tight jars (see § 2.3). The loose soil was not sieved but big clods of earth and debris were removed by hand. 0%, 0.1% and 10%  $\text{C}_2\text{H}_2$  was added to both cores and loose soil immediately after the closure of the lids (§ 2.5). Each treatment was done on 4 replicates (3 replicates for 10%  $\text{C}_2\text{H}_2$ ) for the soil cores and on three replicates for the loose soil, for each soil depth. The jars were incubated at  $25^\circ\text{C}$  ( $\pm 1^\circ\text{C}$ ) in a temperature controlled room. After 2 days, gas samples were taken with 2 ml greased-glass syringes for the analysis of  $\text{N}_2\text{O}$  and  $\text{CO}_2$  (§ 2.4). Immediately

afterwards, the soil cores were broken and soil sub-samples were taken for the analysis of  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , water content,  $\alpha$ -amino-N, soil pH and microbial biomass (see chapter 2 for laboratory methods description); the same analysis were performed on the loose soil samples.

### 3.3 RESULTS AND DISCUSSION

#### 3.3.1 SEASONAL FIELD MEASUREMENT OF $\text{N}_2\text{O}$ FLUXES AND OTHER ENVIRONMENTAL PARAMETERS.

$\text{N}_2\text{O}$  fluxes measured from May '94 to May '95 in the arable field (A) and in the woodland (W) are reported in Figure 3.1.



**Figure 3.1** - Seasonal variations of  $\text{N}_2\text{O}$  fluxes in the arable soil (A) and in the woodland soil (W). Arrows indicate fertilization events (see Table 3.1).

The average mean value, calculated over the entire period of observation, for the arable soil was  $1.4 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$  and it did not differ significantly from the average  $\text{N}_2\text{O}$  flux for the woodland soil ( $1.5 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ).  $\text{N}_2\text{O}$  emissions from the arable soil remained lower than  $4 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$  even when fertilizer was



applied to the field (see Table 3.1 and Figure 3.1). McTaggart *et al.* (1994) reported extremely low fluxes ( $0\text{--}7\text{ g N}_2\text{O-N ha}^{-1}\text{ day}^{-1}$ ) from a winter wheat soil (sandy clay loam) after fertilization ( $180\text{ kg N ha}^{-1}$ ). Skiba *et al.* (1993) reported a maximum  $\text{N}_2\text{O}$  emission rate of  $6.4\text{ g N ha}^{-1}\text{ day}^{-1}$  from a sandy loam soil after fertilization. Skiba *et al.* (1996), measuring  $\text{N}_2\text{O}$  emission from agricultural and seminatural environments in the UK, calculated an average  $\text{N}_2\text{O}$  flux of  $1.04\text{ g N ha}^{-1}\text{ day}^{-1}$ , assuming a mean annual soil  $\text{NO}_3^-$  concentration of  $5\text{ mg N kg soil}^{-1}$ , a mean annual soil temperature of  $10^\circ\text{C}$  and a soil moisture content of 30%. Such a value is in accord with the average value of  $\text{N}_2\text{O}$  fluxes that I measured from both sites, and also with the average soil parameters measured in the field.

For both sites,  $\text{N}_2\text{O}$  fluxes presented a similar seasonal trend. The lowest emissions (almost zero fluxes) were measured around the end of September 1994. In both sites  $\text{N}_2\text{O}$  fluxes increased during late spring, starting from March.

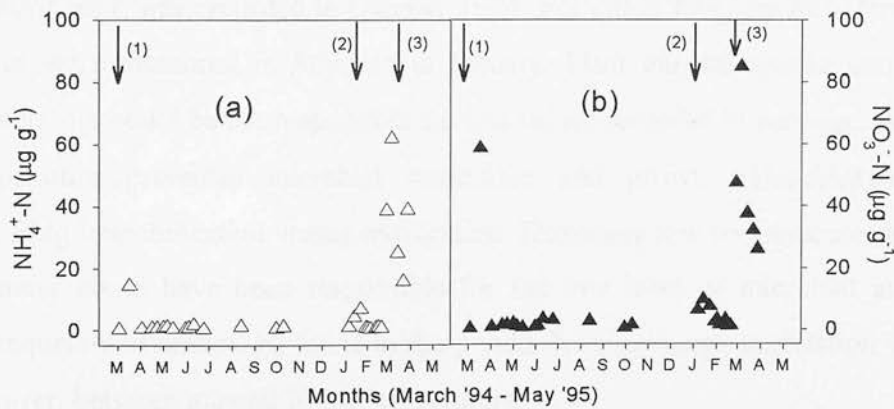
While in the woodland the fluxes were extremely low in February-March, in the arable field a small peak was observed in the same period, probably corresponding to the fertilization application No. 2 (Table 3.1). In May 1995, the very small increase induced by fertilizer application No. 3 (Table 3.1) probably overlapped with the natural increase, as observed in the previous May (notice that in the year 1994 soil was fertilized only in February-March). A small peak was observed in November in both sites. This peak was more pronounced in the woodland, where it was, on average, higher than the fluxes measured during the rest of the year.

Seasonal changes in soil moisture, soil temperature, and C input from plant roots (root exudate, mucilage, sloughed cells, etc.) and crop residues have been found to have a large effect on soil microbial biomass and activity (Ross, 1987). This, in turn, would influence nutrient availability and N transformations in soil, including processes which are the source of  $\text{N}_2\text{O}$  fluxes. In this study,  $\text{N}_2\text{O}$  flux measurements showed high variability and were characterised by high standard errors, so that caution must be taken when interpreting seasonal flux variations.

None of the environmental parameters (mineral N, water content, rain, temperature) measured in the field were found to predict the fluctuations in N<sub>2</sub>O fluxes in both soils. A lack of relationship between N<sub>2</sub>O fluxes and soil moisture, mineral N and soil temperature has been found by other authors (Groffman and Tiedje; 1989a, 1991). However, a lack of any significant relationship could also be due to the very low N<sub>2</sub>O fluxes measured in the soils studied during most of the period.

NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> in the arable soil were extremely low (almost zero) during all the year, except in the arable soil when fertilizer was added (Figure 3.2). During the three events of fertilization the concentrations of NO<sub>3</sub><sup>-</sup>-N were always higher than the concentrations of NH<sub>4</sub><sup>+</sup>-N, suggesting that the NH<sub>4</sub><sup>+</sup> was quickly nitrified. However, it has been observed that when N is added as NH<sub>4</sub>NO<sub>3</sub>, more inorganic N is immobilized as NH<sub>4</sub><sup>+</sup> than as NO<sub>3</sub><sup>-</sup>, and that such immobilization commenced soon after the application of fertilizer (Powlson *et al.*, 1986; Recous and Mary, 1990).

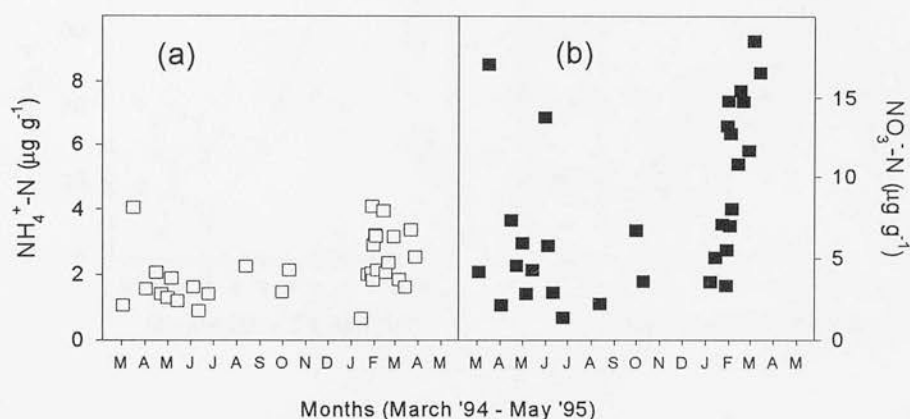
The added N-fertilizer always disappeared in a month or less.



**Figure 3.2** - Seasonal variation of NH<sub>4</sub><sup>+</sup>-N (a) and NO<sub>3</sub><sup>-</sup>-N (b) in the wheat field. Arrows indicated fertilization events (see Table 3.1).

The concentration of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> in the woodland soil was significantly higher than in the arable land (when fertilization induced peaks are excluded) (Figure 3.3). The NH<sub>4</sub><sup>+</sup>-N concentration was never more than about 4 µg N g<sup>-1</sup> soil and the higher

values were recorded in spring (1994 and 1995).  $\text{NO}_3^-$ -N was about four times higher than the  $\text{NH}_4^+$ -N concentration and it reached its maximum in spring (1994 and 1995) as well.



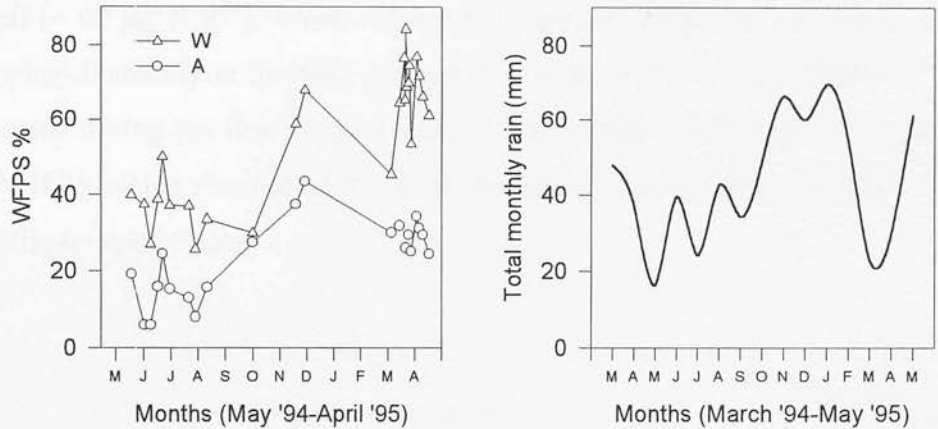
**Figure 3.3** -Seasonal variations of  $\text{NH}_4^+$ -N (a) and  $\text{NO}_3^-$ -N (b) in the woodland.

A minor peak was recorded in October 1994. For either  $\text{NH}_4^+$  or  $\text{NO}_3^-$  the minimum values were measured in July and in January. Plant mineral uptake and increased temperature could be the responsible for low values recorded in summer, as increased temperature promotes microbial respiration and growth (Hendrickson, 1985), favouring immobilization versus nitrification. Extremely low temperatures in January-February could have been responsible for the low level of microbial activity and consequently of mineral N, found in this period. No significant correlation was found, however, between mineral N and temperature.

$\text{NO}_3^-$ -N was significantly correlated with  $\text{NH}_4^+$ -N in both the arable soil ( $P < 0.0001$ ) and the woodland soil ( $P < 0.005$ ).

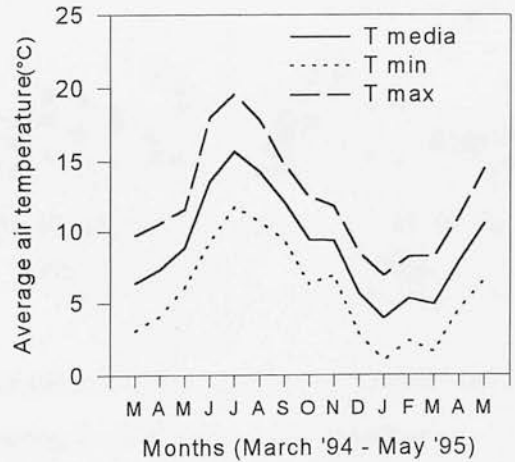
The WFPS % (§ 2.7.6), though much higher in the woodland than in the arable land, had a similar trend in both soils and seasonal variations were comparable to the variation in precipitation (Figure 3.4). Neither WFPS% or precipitation were

correlated with  $\text{N}_2\text{O}$  fluxes, while  $\text{NH}_4^+$  soil concentration in the woodland soil was significantly positively correlated with soil WFPS % ( $P < 0.001$ ).



**Figure 3.4** - Seasonal variations of WFPS % (W-woodland; A-arable land) and total monthly rain at the Gullane site.

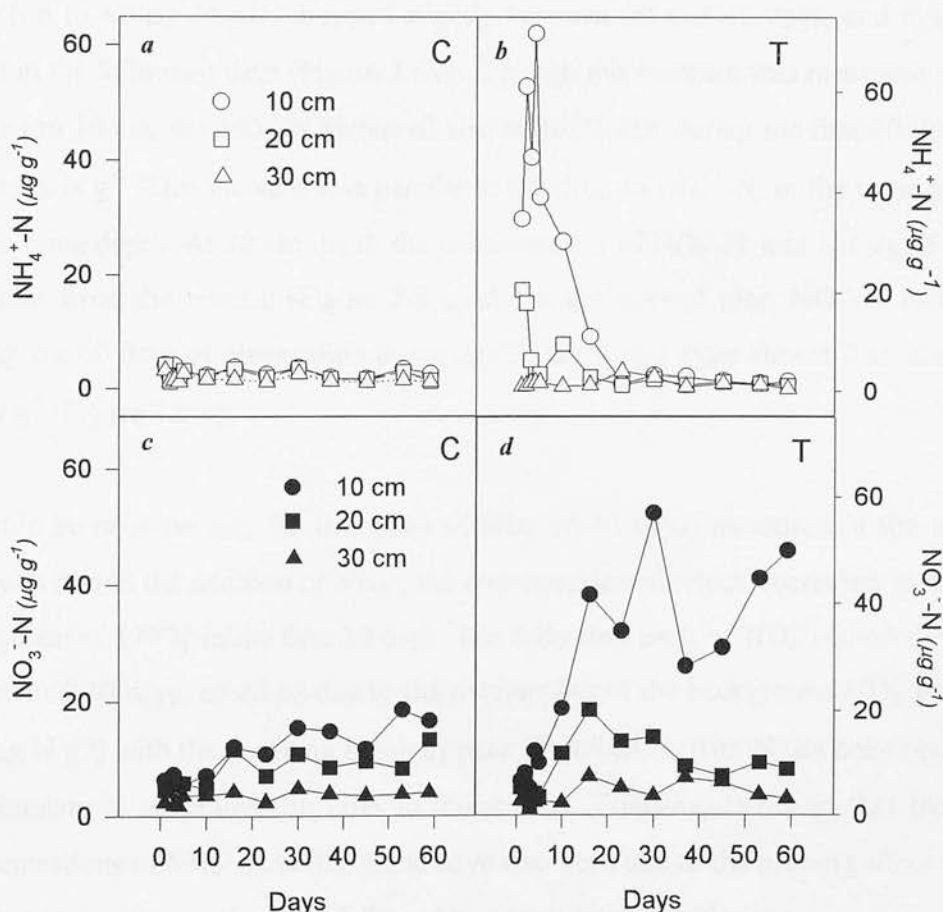
Variations of air temperature from the nearest station in Dunbar are reported in Figure 3.5. Air temperature resulted correlated significantly in both sites with soil temperature. In neither of the two sites, could temperature variations explain the variations in  $\text{N}_2\text{O}$  fluxes.



**Figure 3.5** Air temperature in Dunbar (monthly averages).

### 3.3.2 II - N<sub>2</sub>O FLUXES AND MINERAL N TRANSFORMATIONS IN SOIL, FOLLOWING (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> FERTILISATION OF THE WOODLAND SOIL.

Addition of ammonium sulphate in the treated plot resulted in an increase of NH<sub>4</sub><sup>+</sup>-N in soil (~ 60 µg N g<sup>-1</sup>), which remained at high concentrations for almost 10 days, dropping drastically in the following 20 days (Figure 3.6 b). The values of NH<sub>4</sub><sup>+</sup>-N measured during the first 10 days showed a very high standard error (*Appendix II*, Table II.1), which was probably due to the uneven distribution of fertilizer obtained with the spraying system.



**Figure 3.6** - Values of mineral N measured in the treated (T) and in the control (C) plot at 3 depths during the first 60 days after fertilization.

$\text{NH}_4^+$  remained mainly concentrated in the top 10 cm of soil and only a small fraction of the added N reached 10-20 cm depth at the beginning of the fertilization (increasing the concentration to  $\sim 20 \mu\text{g N g}^{-1}$ ), declining to the control level after about 30 days (Figure 3.6 *a, b*). This increase of  $\text{NH}_4^+$ -N at 10-20 cm was probably the result of water transport, more than any biological transformation, as the level of  $\text{NH}_4^+$ -N at 10-20 cm was found to be predicted by a linear combination of daily precipitations (36.1% of contribution to the prediction) and  $\text{NH}_4^+$ -N concentration at 0-10 cm (30.8 %). At 20-30 cm the addition of fertilizer did not induce any increase of  $\text{NH}_4^+$ -N throughout the 60 days of measurements.

$\text{NO}_3^-$ -N concentration in the treated plot increased quite sharply during the first 30 days (up to  $60 \mu\text{g N g}^{-1}$ ), dropped slightly between 30 and 40 days, and increased again in the following days (Figure 3.6 *d*). Though this increase was measured mainly in the top 10 cm, the  $\text{NO}_3^-$ -N increased also at 10-20 cm, during the first 20 days, up to  $20 \mu\text{g N g}^{-1}$ . This increase was parallel to the drop in  $\text{NH}_4^+$ -N, in the same period, at the same depth. At 30 cm depth the concentration of  $\text{NO}_3^-$ -N was not significantly different from the control (Figure 3.6 *c, d*). In the control plot,  $\text{NO}_3^-$ -N increased during the 60 days of observation in the top 20 cm, going from almost 0 to about  $20 \mu\text{g N g}^{-1}$  (Figure 3.6 *c*).

It could be possible that the first peak of  $\text{NO}_3^-$  (0-30 days) measured in the treated plot was due to the addition of  $\text{NH}_4^+$ , the concentration of which decreased parallel to the increase of  $\text{NO}_3^-$  in the first 30 days. The following peak of  $\text{NO}_3^-$  (40-60 days), in the second 30 days, could be due to the overlapping of the background  $\text{NO}_3^-$  increase ( $20 \mu\text{g N g}^{-1}$ ) with the declining previous peak. Fertilization with N has been observed to stimulate N mineralization rates in forest soils (Williams, 1972) so that the high concentrations of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  could have also been due to the priming effect of the fertilizer on soil mineralization, followed by a very active nitrification.

The  $\text{NH}_4^+$ -N measured in the treated plot at 0-10, 10-20 and 20-30 cm depth did not correlate directly with  $\text{NO}_3^-$ -N measured at the same depth. On the contrary, a significant negative correlation was found between  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N over the 0-30



cm zone. 58% of the variance in  $\text{NO}_3^-$ -N concentration in the treated plot was accounted for by the  $\text{NH}_4^+$ -N concentration and 27% by the WFPS%. For the control plot no significant correlation was found between the  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N concentrations in the soil. However, both  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N measured at 0-10 cm were correlated positively and significantly with  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N measured at 10-20 cm depth.

The  $\text{N}_2\text{O}$  fluxes measured from the treated plot ( T ) were significantly higher than the fluxes from the control plot ( C ), measured with both the automated (A) and the manual (M) chambers; some statistical parameters for the overall period of observation, based on daily averages, are reported in Table 3.2.

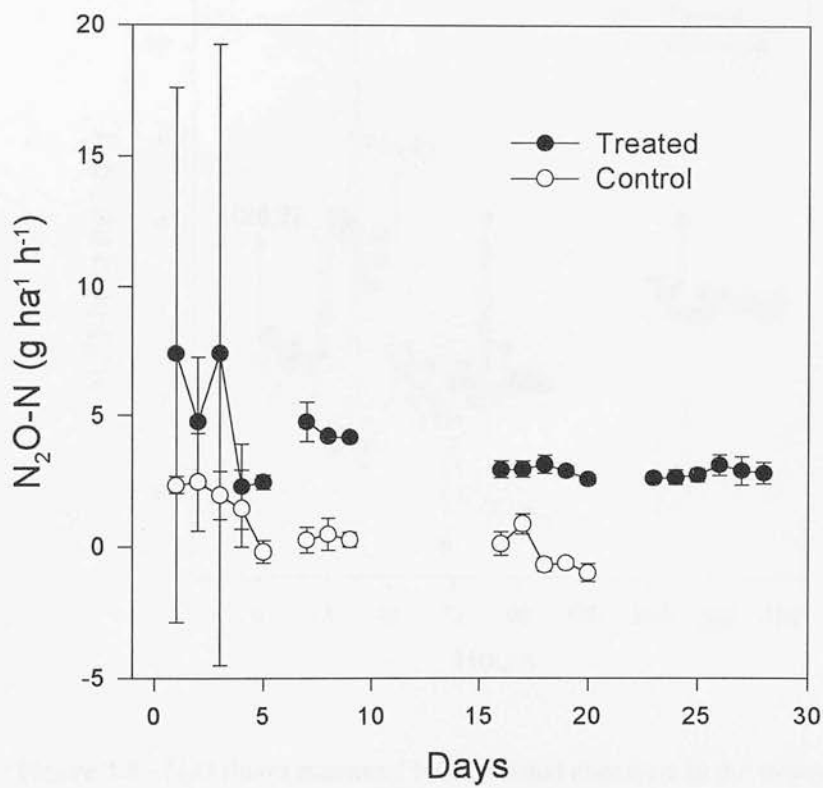
**Table 3.2** - Some statistical parameters calculated on a time series population of  $\text{N}_2\text{O}$  daily averages, measured in the treated and in the control plot with two different type of chambers.

	<i>MEAN</i>	<i>MED</i>	<i>MIN</i>	<i>MAX</i>	<i>STDEV</i>	<i>STERR</i>	<i>t-Test</i>
<b>TA</b>	3.99	3.14	2.29	7.38	1.72	0.48	P<0.0001
<b>CA</b>	0.60	0.27	-0.98	2.47	1.15	0.32	
<b>TM</b>	1.08	1.11	0.50	1.63	0.34	0.11	P<0.005
<b>CM</b>	0.59	0.58	0.28	1.34	0.34	0.11	

Note: T: Treated, C: Control (Plots); A: automated, M: Manual (chambers).

It is apparent from Table 3.2 that the distribution of the population of  $\text{N}_2\text{O}$  measurements taken by automated chambers was more skewed than the population of samples taken by manual chambers, both in the treated and untreated plots, as indeed the difference between the mean and the median values was much higher for the former population than for the latter. This shows a much higher variability in samples taken by automated chambers. In Figure 3.7 the values are reported of the fluxes measured in the treated and in the control plots by automated chambers. The highest fluxes in the treated plot were measured in the first 5 days, reaching an average of about  $7\text{-}8 \text{ g N ha}^{-1} \text{ d}^{-1}$ ; in the following 10 days the flux declined and then remained stable around  $3 \text{ g N ha}^{-1} \text{ d}^{-1}$  for the next 15 days. For every sampling occasion, the

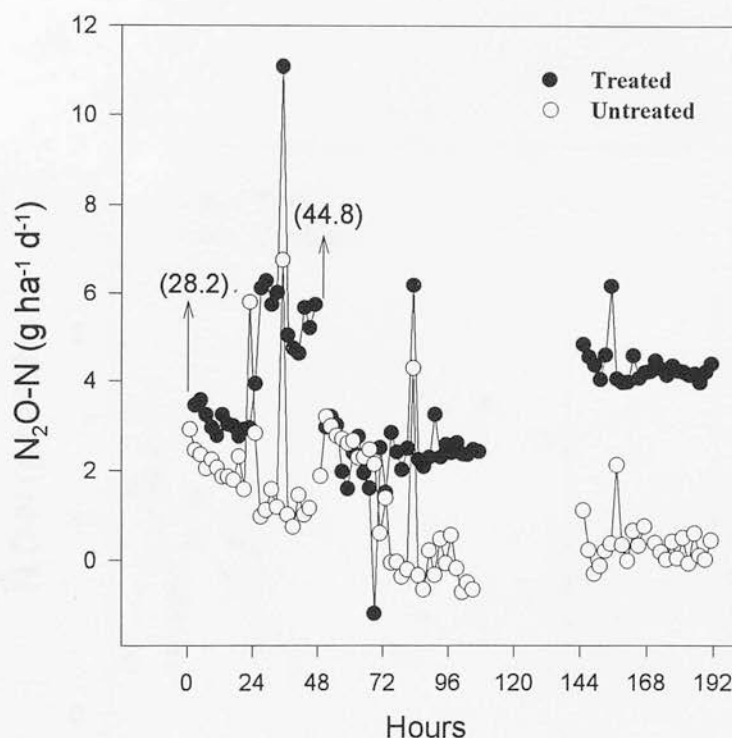
flux from the treated plot was higher than the flux from the control plot. A very high variability can be noticed for the values of  $\text{N}_2\text{O}$  emissions measured in the treated plot during the first 5 days. A detail of the first 8 days measurements is given in Figure 3.8, where are reported all the single samples taken hour by hour with the autochambers.



**Figure 3.7** - Daily average  $\text{N}_2\text{O}$  fluxes measured by automated chambers in the treated (T) and in the control (C) plots, in 30 days following the fertilization.

For the first 8 days, samples were taken every two hours. This high frequency of sampling made it possible to measure a few, very high, peaks, which explain the extremely high variation around the average presented in Figure 3.7 (bars represent one standard deviation). From day 15 to day 30, gas was sampled 4 times a day

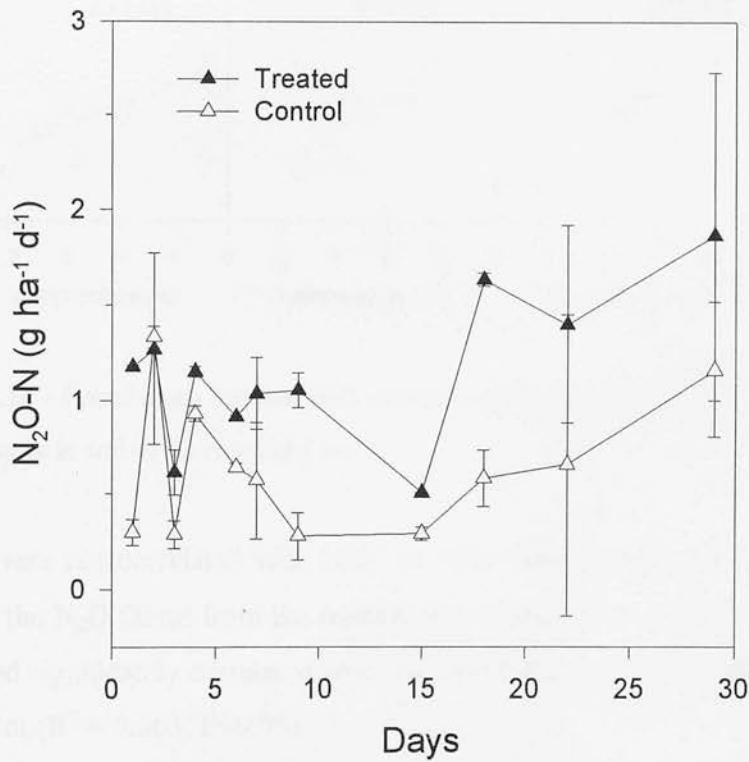
(every 6 hours). The lack of big peaks of  $\text{N}_2\text{O}$  in this period (Figure 3.7) could either be due to the lower sampling frequency or to a real lack of high  $\text{N}_2\text{O}$  emissions rates. The control plot never showed peaks much higher than  $6 \text{ g N ha}^{-1} \text{ d}^{-1}$ , suggesting the extremely high peaks measured in the treated soil were due to the fertilization.



**Figure 3.8** -  $\text{N}_2\text{O}$  fluxes measured by automated chambers in the treated and in the control plot during the first 8 days after fertilization. Values in brackets are off scale.

$\text{N}_2\text{O}$  fluxes measured by manual chambers were significantly higher in the treated plot than in the control plot (Figure 3.9), though the fluxes measured in the treated plot by manual chambers were much lower than the fluxes measured by automated chambers. The number of chambers used in the experiment was not sufficient to demonstrate statistically that such a difference depended on the sampling technique, so that it cannot be excluded that the automated chamber in the treated plot was located on a spot of higher microbial activity.

Fluxes measured by manual chambers were quite variable (Figure 3.9); however, the variations in the control and in the treated plot were generally very similar. This suggests that, though fertilization stimulated  $\text{N}_2\text{O}$  production in soil, the processes which were producing  $\text{N}_2\text{O}$  in the control and in the treated plot, and were measured by manual chambers, were equally influenced by environmental factors. A slight increase in the  $\text{N}_2\text{O}$  flux was noticed from day 1 to day 30.

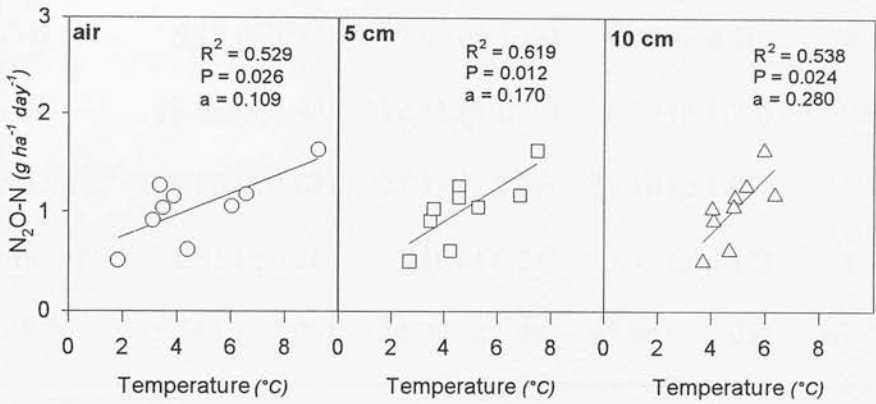


**Figure 3.9** -  $\text{N}_2\text{O}$  fluxes measured in the treated and in the control plots by manual chambers.

This increase could be explained as a general influence of the temperature on microbial processes.  $\text{N}_2\text{O}$  emissions from the treated plot were, in fact, correlated significantly with temperature measured in air and at 5 and 10 cm depth, though the best fit was obtained with the temperature measured at 5 cm depth (Figure 3.10), where the variation was intermediate between that of the air temperature and the

temperature at 10 cm depth. Temperature was not correlated with  $\text{NH}_4^+$  or  $\text{NO}_3^-$  variation in soil, either in the treated or in the control plot.

$\text{N}_2\text{O}$  fluxes measured by automated chambers were not correlated with temperature, either in the treated or in the control plots.



**Figure 3.10** - Correlations between  $\text{N}_2\text{O}$  emissions and temperature at 0, 5 and 10 cm depth in soil in the woodland site.

$\text{N}_2\text{O}$  fluxes were not correlated with  $\text{NH}_4^+$  or  $\text{NO}_3^-$  concentrations in soil, with the exception of the  $\text{N}_2\text{O}$  fluxes from the treated plot measured by automated chambers, which resulted significantly correlated with the total  $\text{NH}_4^+$  concentration (0-30 cm) in the treated plot ( $R^2 = 0.863$ ,  $P < 0.05$ ).

### 3.3.3 III - APPLICATION OF A SOIL CORE METHOD TO INVESTIGATE THE RELATIONSHIP BETWEEN $\text{N}_2\text{O}$ FLUXES AND SOIL PARAMETERS.

Overall, the woodland soil showed a much higher microbial biomass and activity than the arable soil. Average values of gas fluxes and several soil parameters measured in the woodland soil cores and loose soil samples are reported in Table 3.3 and Table 3.4, respectively. Most of the microbial activity was concentrated in the top 5 cm, and already at a depth of 5-10 cm all the measured variables decreased by about one third. Such a decrease was less pronounced passing to 15 and to 20 cm depth.

**Table 3.3** - Soil parameters measured in the woodland soil cores from 0 to 20 cm. Values in brackets are one standard error.

	Soil depth (cm)			
	0 -5	5 -10	10 - 15	15 - 20
<b>N<sub>2</sub>O-N flux</b> <i>ng g<sup>-1</sup> h<sup>-1</sup></i>	1.85 (± 1.57)	0.35 (± 0.25)	0.08 (± 0.00)	0.08 (± 0.00)
<b>CO<sub>2</sub> flux</b> <i>μg g<sup>-1</sup> h<sup>-1</sup></i>	8.36 (± 1.00)	5.13 (± 0.87)	3.18 (± 0.44)	2.45 (± 0.37)
<b>NH<sub>4</sub><sup>+</sup>-N</b> <i>μg g<sup>-1</sup></i>	5.15 (± 2.45)	2.63 (± 2.11)	0.48 (± 0.18)	0.35 (± 0.21)
<b>NO<sub>3</sub><sup>-</sup>-N</b> <i>μg g<sup>-1</sup></i>	18.08 (± 3.48)	12.11 (± 1.50)	8.84 (± 1.76)	9.63 (± 0.80)
<b>*Biomass<sub>mic</sub></b>	39.65 (± 4.07)	27.51 (± 2.2)	21.16 (± 1.99)	17.99 (± 0.77)
<b>α-amino-N</b> <i>μg g<sup>-1</sup></i>	1.62 (± 0.56)	1.16 (± 0.34)	1.35 (± 0.12)	1.06 (± 0.02)
<b>WFPS %</b>	57.61 (± 2.06)	54.02 (± 1.49)	45.68 (± 2.05)	43.10 (± 1.48)

\* μg α-amino-N g<sup>-1</sup>

**Table 3.4** - Soil parameters measured in the loose woodland soil from 0 to 20 cm. Values in brackets are one standard error.

	Soil depth (cm)			
	0 -5	5 -10	10 - 15	15 - 20
<b>N<sub>2</sub>O-N flux</b> <i>ng g<sup>-1</sup> h<sup>-1</sup></i>	0.42 (± 0.21)	0.22 (± 0.03)	0.14 (± 0.02)	0.16 (± 0.00)
<b>CO<sub>2</sub> flux</b> <i>μg g<sup>-1</sup> h<sup>-1</sup></i>	7.62 (± 1.34)	2.70 (± 0.12)	1.54 (± 0.89)	1.86 (± 0.51)
<b>NH<sub>4</sub><sup>+</sup>-N</b> <i>μg g<sup>-1</sup></i>	1.71 (± 0.10)	1.42 (± 0.30)	0.78 (± 0.13)	0.41 (± 0.03)
<b>NO<sub>3</sub><sup>-</sup>-N</b> <i>μg g<sup>-1</sup></i>	20.71 (± 0.51)	12.26 (± 0.13)	13.07 (± 0.85)	11.47 (± 0.31)
<b>*Biomass<sub>mic</sub></b>	48.32 (± 0.43)	34.13 (± 0.49)	27.62 (± 0.26)	21.68 (± 0.40)
<b>α-amino-N</b> <i>μg g<sup>-1</sup></i>	4.04 (± 0.17)	3.29 (± 0.30)	2.49 (± 0.12)	1.74 (± 0.09)
<b>pH</b>	7.88 (± 0.02)	8.05 (± 0.01)	8.13 (± 0.003)	8.20 (± 0.01)

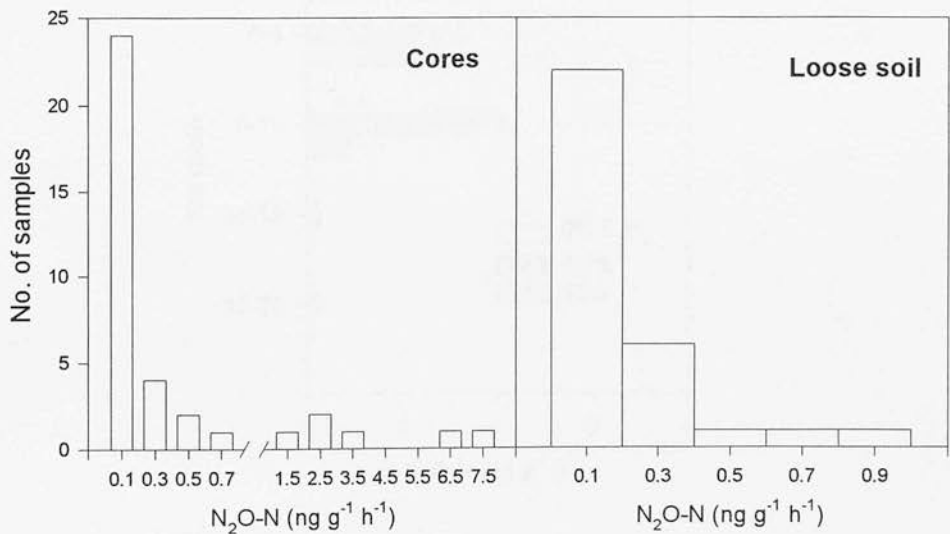
\* μg α-amino-N g<sup>-1</sup>



The standard error was generally much higher for the parameters measured in the soil cores as compared with the loose soil, especially for the top 10 cm (Table 3.3 and Table 3.4). Big clods and organic matter (fresh debris) were removed from the loose soil before incubation. This suggests that the freshly decomposing organic matter could have been mainly responsible for the high variability found for the different parameters in the soil cores.

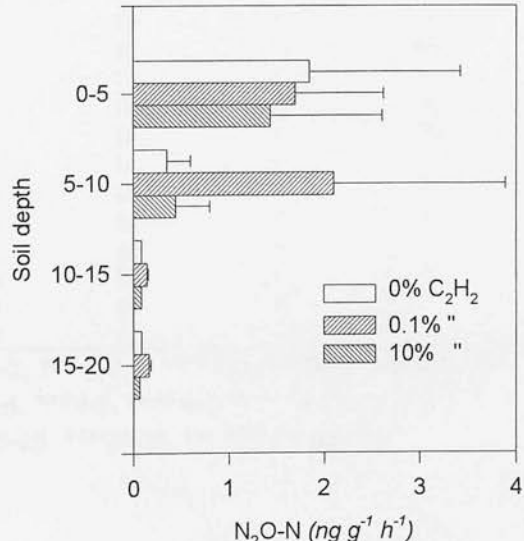
Average  $\text{N}_2\text{O}$  emissions, measured from cores from the top 5 cm, were  $1.85 \text{ ng g}^{-1} \text{ h}^{-1}$ , almost 5 times higher than the emissions from the loose soil at same depth.  $\text{N}_2\text{O}$  emissions decreased with depth much more smoothly in the loose samples than in the cores, probably reflecting a more uniform distribution of mineral N and organic matter in the loose soil (Table 3.3 and Table 3.4). Parkin, (1987), in a similar experiment on soil cores incubated in aerobic conditions with no amendments, found that the  $\text{N}_2\text{O}$  production from 16 cm length cores was generally concentrated in the top 5 cm. Further analysing the content of those cores, he found that emissions were mainly associated with organic debris.

Estimates of  $\text{N}_2\text{O}$  emission rates from soil cores were highly variable and displayed a highly skewed distribution (Figure 3.11).



**Figure 3.11** -  $\text{N}_2\text{O}$  fluxes from woodland soil cores and loose soil samples.

It is apparent that the high variability in the cores was due to a few samples having high emission rates. The median rate ( $0.1 \text{ ng N}_2\text{O-N g}^{-1} \text{ h}^{-1}$ ) was about 8 times less than the mean rate ( $0.8 \text{ ng N}_2\text{O-N g}^{-1} \text{ h}^{-1}$ ) and the standard deviation was extremely high ( $1.72 \text{ ng N}_2\text{O-N g}^{-1} \text{ h}^{-1}$ ). The same was not observed for the  $\text{N}_2\text{O}$  emission rates from the loose soil samples (mean = 0.22, median = 0.16, Std Dev = 0.16) (Figure 3.11). The high rates of activity measured in soil cores could be due to a non-homogeneous dispersion of fresh organic matter, and could have been further favoured by the more structured soil in the cores. At the end of the present experiment, all the soil cores were broken and the presence of fresh organic matter was recorded. The numbers of occasions when high  $\text{N}_2\text{O}$  fluxes from the woodland soil cores (which was the only soil to exhibit a high level of  $\text{N}_2\text{O}$  production), were associated with high amounts of  $\text{NH}_4^+$  (Table 3.5) resulted significantly higher ( $P = 0.01$ ) than the number of occasions when  $\text{N}_2\text{O}$  fluxes were associated with high concentrations of  $\text{NO}_3^-$ , and in 86% of the cases  $\text{N}_2\text{O}$  fluxes were associated with the presence of organic debris.  $\text{N}_2\text{O}$  results for the soils cores incubated with 0, 0.1% and 10%  $\text{C}_2\text{H}_2$  are shown in Figure 3.12.



**Figure 3.12** -  $\text{N}_2\text{O}$  fluxes from woodland cores with 0, 0.1 and 10 %  $\text{C}_2\text{H}_2$ .

**Table 3.5-** Presence of organic debris (+) and exceptionally high contents of  $\text{NH}_4^+\text{-N}$  or  $\text{NO}_3^-\text{-N}$  in the woodland soil cores.

Core n°	Soil depth (cm)	% $\text{C}_2\text{H}_2$	$\text{N}_2\text{O-N}$	$\text{NH}_4^+\text{-N}$	$\text{NO}_3^-\text{-N}$	Organic debris
1	0-5	0	*	*	*****	-
2	0-5	0	**	*****	*	+
3	0-5	0	*****	*****	*****	+
4	0-5	0	*	*	*	-
5	5-10	0	*	*	*	-
6	5-10	0	*	*	*	-
7	5-10	0	*	*	**	-
8	5-10	0	**	*****	*	+
9	10-15	0	*	*	*	-
10	10-15	0	*	*	*	-
11	10-15	0	*	*	*	-
12	10-15	0	*	*	*	-
13	15-20	0	*	*	*	-
14	15-20	0	*	*	*	-
15	15-20	0	*	*	*	-
16	0-5	0.1	*	*	*	-
17	0-5	0.1	***	*	*	-
18	0-5	0.1	*	**	*	-
19	0-5	0.1	*****	*	*	+
20	5-10	0.1	*	***	*	-
21	5-10	0.1	*	*	*	-
22	5-10	0.1	*	*	*	-
23	5-10	0.1	*****	*****	*	+
24	10-15	0.1	*	*	*	-
25	10-15	0.1	*	*	*	-
26	10-15	0.1	*	*	*	-
27	10-15	0.1	*	*	*	-
28	15-20	0.1	*	*	*	-
29	15-20	0.1	*	*	*	-
30	15-20	0.1	*	*	*	-
31	0-5	10	*	*	*	-
32	0-5	10	***	***	*	+
33	0-5	10	*	*	*	-
34	5-10	10	*	*	*	-
35	5-10	10	*	*	*	-
36	5-10	10	*	*	*	-
37	10-15	10	*	*	*	-
38	10-15	10	*	*	*	-
39	10-15	10	*	*	*	-
40	15-20	10	*	*	*	-
41	15-20	10	*	*	*	-
42	15-20	10				-

$\text{N}_2\text{O}$ : \* < 0.5, \*\* 0.5-2, \*\*\* 2-3.5, \*\*\*\*3-6, \*\*\*\*\*6-12  $\text{ng N g}^{-1} \text{ h}^{-1}$

$\text{NH}_4^+\text{-N}$ : \* 0-2, \*\*2-4, \*\*\*4-6, \*\*\*\*6-8, \*\*\*\*\*8-12  $\mu\text{g N g}^{-1}$

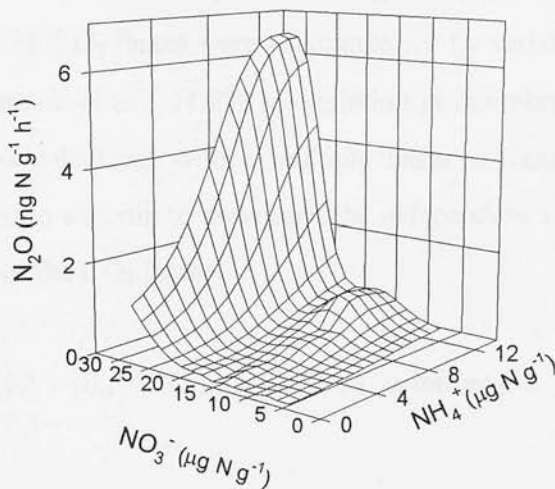
$\text{NO}_3^-\text{-N}$ : \* <15, \*\* 15-20, \*\*\*20-25, \*\*\*\*25-30  $\mu\text{g N g}^{-1}$

The extremely high variability did not allow a clear distinction of the sources of  $\text{N}_2\text{O}$ . The addition of 0%, 0.1% and 10%  $\text{C}_2\text{H}_2$  did not seem to produce significant differences in  $\text{N}_2\text{O}$  emissions. The big peak recorded at 5-10 cm with 0.1%  $\text{C}_2\text{H}_2$ ,

could be only explained by the presence of a high denitrifying activity, associated with the presence of organic matter, or with heterotrophic nitrifying activity, both of which are insensitive to low concentrations of  $C_2H_2$ . High fluxes and big variability disappeared with increasing soil depth.

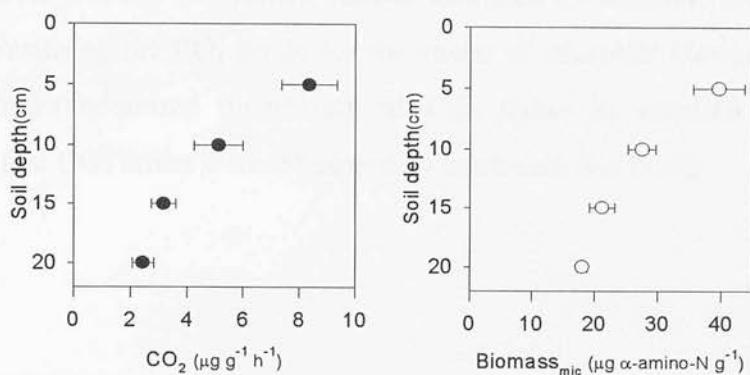
Emissions of  $N_2O$  from loose soil with different concentrations of  $C_2H_2$  were generally very low and no significant difference was found between 0%, 0.1% and 10%  $C_2H_2$  (values reported in Table II.2 of *Appendix II*).

$N_2O$  fluxes from soil cores steeply increased for increasing concentrations of  $NH_4^+$ , whereas a less pronounced increase of  $N_2O$  production was observed for increasing concentrations of  $NO_3^-$  (Figure 3.13); however, the biggest  $N_2O$  emissions corresponded to high concentrations of total mineral N ( $NH_4^+$  plus  $NO_3^-$ ).



**Figure 3.13** -  $N_2O$  flux variations as a function of  $NH_4^+$  and  $NO_3^-$  variations, calculated from the woodland soil core results.

The microbial respiration, measured as  $CO_2$  flux ( $\mu g\ g^{-1}\ h^{-1}$ ), did not differ significantly between soil cores and loose soil (Table 3.3 and Table 3.4).  $CO_2$  flux did not decrease linearly with depth but it had a pronounced parabolic trend which was very similar to the trend of microbial biomass (Figure 3.14) from 0 to 20 cm depth.



**Figure 3.14** - Microbial respiration and biomass variation with soil depth, measured in the woodland soil cores.

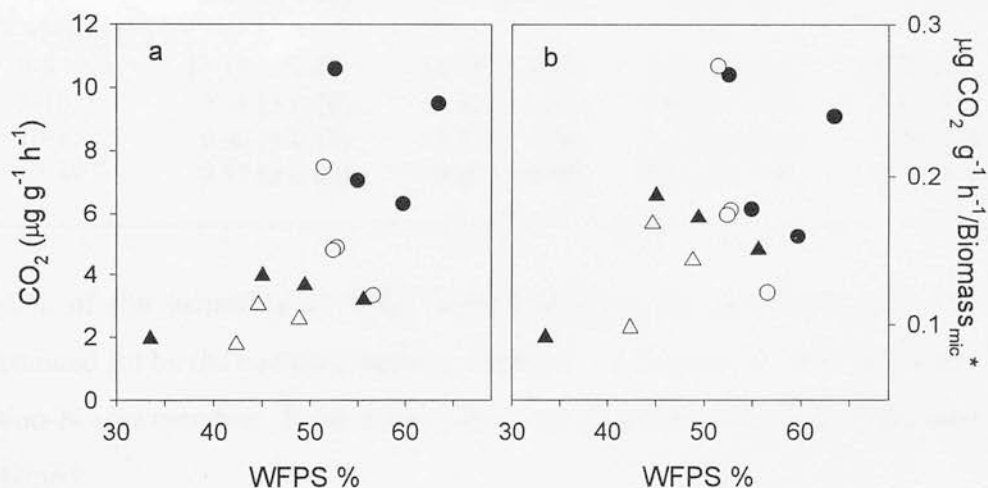
Indeed, microbial biomass and CO<sub>2</sub> flux were found to be significantly positively correlated ( $P < 0.001$ ,  $R^2 = 0.597$ ). Considering all the measured parameters, 59.7% of the variance in the CO<sub>2</sub> fluxes were accounted for by variation in NH<sub>4</sub><sup>+</sup>-N, 10.9% by variation in α-amino-N and 11.8% by variation in microbial biomass. The following relationship was calculated with a multiple linear regression after that a forward stepwise regression was run to determine the independent variables that produced the best prediction of the CO<sub>2</sub> fluxes:

$$CO_2 = -1.92 + (0.57 \text{ NH}_4^+\text{-N}) + (2.25 \text{ α-amino-N}) + (0.096 \text{ biomass}_{mic})$$

$$(R^2 = 0.82)$$

The equation includes the main parameters which can be expected to influence microbial activity in soil such as the microbial biomass size, the presence of organic matter and concentration of available N, which stimulates microbial growth and activity but also reflects soil mineralization activity. CO<sub>2</sub> flux apparently increased for increase of water filled pore space from 30% to about 65% (Figure 3.15a, b). Skopp *et al.* (1990) demonstrated, in laboratory experiments, that microbial respiration increases generally in this same range. However, caution must be taken in interpreting the data because though the highest values of CO<sub>2</sub> fluxes were measured at high water

contents they also corresponded with the cores taken in the first cm of soil (Fig. 3.15a) where also other parameters such as microbial biomass and  $\alpha$ -amino-N were higher. Normalizing the  $\text{CO}_2$  fluxes for the values of microbial biomass (Fig. 3.15b) gave a more randomized distribution of  $\text{CO}_2$  fluxes as function of WFPS % confirming that  $\text{CO}_2$  fluxes were influenced by more than one factor.



**Figure 3.15** - Relation between the WFPS % and the  $\text{CO}_2$  flux ( $\mu\text{g g}^{-1} \text{h}^{-1}$ ) (a) and (b) the ratio  $\text{CO}_2$  flux to microbial biomass ( $\mu\text{g } \alpha\text{-amino-N g}^{-1}$ ), measured in the woodland soil cores at different depths (● 0-5 cm, ○ 5-10 cm, ▲ 10-15 cm, △ 15-20 cm).

Mineral N content was generally not significantly different between the soil cores and in the loose soil samples; however, cores concentrations of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  showed a much higher variability (see Table 3.3 and Table 3.4). Addition of  $\text{C}_2\text{H}_2$  to the soil cores drastically reduced the production of  $\text{NO}_3^-$ , which was significantly lower after 24 h than the  $\text{NO}_3^-$  measured in soil at time zero (Table 3.6). Part of the initial  $\text{NO}_3^-$  could have been either denitrified or immobilized (Recous and Mary, 1990). Addition of  $\text{C}_2\text{H}_2$  did not result in any significant variation in the  $\text{NH}_4^+$  concentration at 0-5 cm and 5-10 cm depth, while at 10-15 cm and 15-20 cm,  $\text{NH}_4^+$  increased significantly either with 0.1% or 10%  $\text{C}_2\text{H}_2$  (Table 3.6).



**Table 3.6** - Values of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  extracted from the woodland soil cores after 48 h incubation in presence of 0%, 0.1% (100 Pa) and 10% (10 kPa)  $\text{C}_2\text{H}_2$ . In brackets is reported one standard error.

		Concentration of $\text{NH}_4^+$ and $\text{NO}_3^-$ ( $\mu\text{g N g}^{-1}$ soil)			
		<i>Initial value(T<sub>0</sub>)</i>	<i>0 % C<sub>2</sub>H<sub>2</sub></i>	<i>0.1% C<sub>2</sub>H<sub>2</sub></i>	<i>10% C<sub>2</sub>H<sub>2</sub></i>
$\text{NH}_4^+\text{-N}$ ( $\mu\text{g g}^{-1}$ )					
0-5 cm		1.98 ( $\pm 0.03$ )	5.15 ( $\pm 2.45$ )	3.18 ( $\pm 1.12$ )	4.00 ( $\pm 1.84$ )
5-10 "		1.15 ( $\pm 0.03$ )	2.63 ( $\pm 2.11$ )	3.39 ( $\pm 1.54$ )	1.95 ( $\pm 0.33$ )
10-15 "		0.68 ( $\pm 0.00$ )	0.48 ( $\pm 0.18$ )	1.61 ( $\pm 0.41$ )	1.26 ( $\pm 0.38$ )
15-20 "		0.45 ( $\pm 0.03$ )	0.35 ( $\pm 0.21$ )	1.44 ( $\pm 0.46$ )	1.26 ( $\pm 0.03$ )
$\text{NO}_3^-\text{-N}$ ( $\mu\text{g g}^{-1}$ )					
0-5 cm		13.14 ( $\pm 0.88$ )	18.08 ( $\pm 3.48$ )	4.37 ( $\pm 1.09$ )	6.20 ( $\pm 1.23$ )
5-10 "		9.58 ( $\pm 0.16$ )	12.12 ( $\pm 1.50$ )	5.05 ( $\pm 2.11$ )	4.03 ( $\pm 0.31$ )
10-15 "		9.90 ( $\pm 0.18$ )	8.84 ( $\pm 1.76$ )	5.10 ( $\pm 1.16$ )	3.24 ( $\pm 1.06$ )
15-20 "		9.57 ( $\pm 0.03$ )	9.63 ( $\pm 0.80$ )	3.99 ( $\pm 1.19$ )	4.71 ( $\pm 1.43$ )

33.3% of the variability of  $\text{NH}_4^+$  concentration in the woodland soil cores was accounted for by the microbial activity, measured as respiration, and 49.8% by the  $\alpha$ -amino-N concentration. From a multilinear regression the following relationship was obtained:

$$\text{NH}_4^+\text{-N} = 2.71 + (0.98 \text{ CO}_2) - (4.01 \text{ } \alpha\text{-amino-N}) \quad (R^2 = 0.831)$$

52.8% of the variability in  $\text{NO}_3^-$  concentration in the woodland soil cores was accounted for by the concentration of  $\text{NH}_4^+$ , and 23.8% by the size of the microbial population. The following relationship was obtained:

$$\text{NO}_3^-\text{-N} = 2.56 + (0.82 \text{ NH}_4^+\text{-N}) + (0.29 \text{ biomass}_{\text{mic}}) \quad (R^2 = 0.766)$$

In Table 3.7 and Table 3.8 the values are reported of the gas fluxes and soil parameters measured in the wheat field soil cores and loose soil, respectively. In the majority of cases the two different soil incubations did not give significantly different results.

**Table 3.7** - Soil parameters measured from 0 to 20 cm in the wheat field soil cores. Values in brackets are one standard error.

	Soil depth (cm)			
	0 -5	5 -10	10 - 15	15 - 20
<b>N<sub>2</sub>O-N flux</b> <i>ng g<sup>-1</sup> h<sup>-1</sup></i>	0.05 (± 0.01)	0.04 (± 0.01)	0.04 (± 0.00)	0.04 (± 0.01)
<b>CO<sub>2</sub> flux</b> <i>μg g<sup>-1</sup> h<sup>-1</sup></i>	3.67 (± 1.14)	4.26 (± 0.90)	2.66 (± 1.01)	2.28 (± 0.51)
<b>NH<sub>4</sub><sup>+</sup>-N</b> <i>μg g<sup>-1</sup></i>	0.38 (± 0.10)	0.46 (± 0.12)	0.51 (± 0.11)	0.41 (± 0.15)
<b>NO<sub>3</sub><sup>-</sup>-N</b> <i>μg g<sup>-1</sup></i>	1.29 (± 0.25)	2.27 (± 0.18)	2.49 (± 0.23)	2.71 (± 0.47)
<b>*Biomass<sub>mic</sub></b>	9.11 (± 1.59)	9.48 (± 1.12)	8.27 (± 1.19)	7.37 (± 1.12)
<b>α-amino-N</b> <i>μg g<sup>-1</sup></i>	0.77 (± 0.06)	0.85 (± 0.14)	0.81 (± 0.15)	0.55 (± 0.08)
<b>WFPS %</b>	33.79 (± 1.12)	32.26 (± 1.16)	31.80 (± 1.17)	30.77 (± 1.21)

\* μg α-amino-N g<sup>-1</sup>

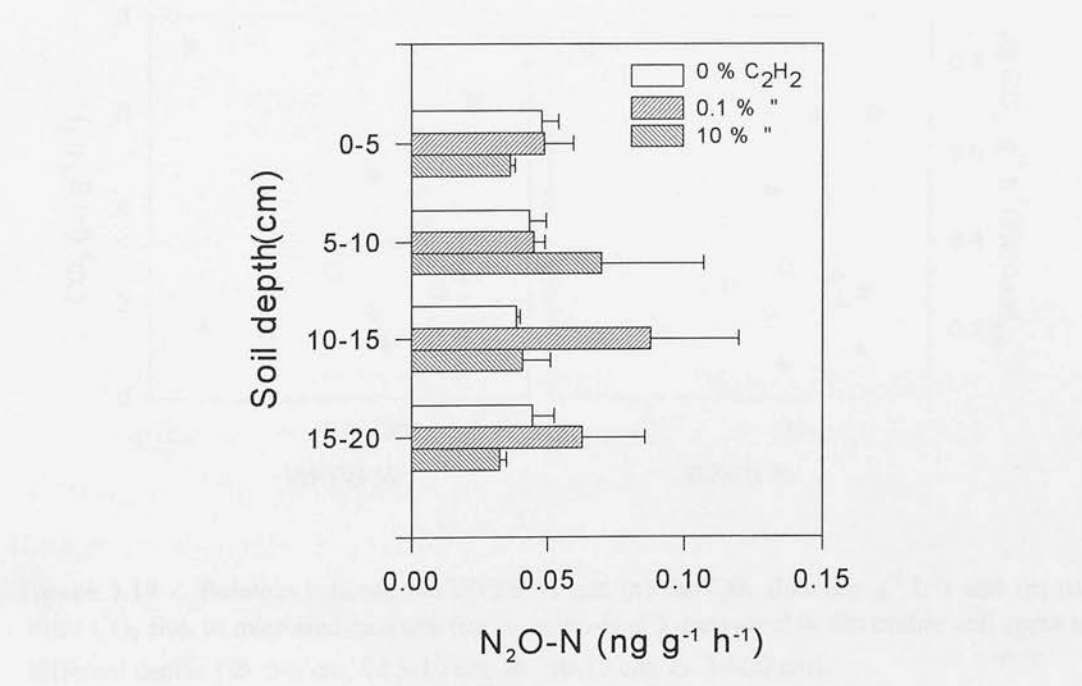
**Table 3.8** - Soil parameters measured from 0 to 20 cm in the loose wheat field soil. Values in brackets are one standard error.

	Soil depth (cm)			
	0 -5	5 -10	10 - 15	15 - 20
<b>N<sub>2</sub>O-N flux</b> <i>ng g<sup>-1</sup> h<sup>-1</sup></i>	0.08 (± 0.02)	0.08 (± 0.00)	0.07 (± 0.00)	0.07 (± 0.00)
<b>CO<sub>2</sub> flux</b> <i>μg g<sup>-1</sup> h<sup>-1</sup></i>	5.10 (± 1.40)	3.00 (± 0.70)	4.31 (± 0.56)	1.61
<b>NH<sub>4</sub><sup>+</sup>-N</b> <i>μg g<sup>-1</sup></i>	0.30 (± 0.07)	0.22 (± 0.06)	0.22 (± 0.00)	0.59 (± 0.07)
<b>NO<sub>3</sub><sup>-</sup>-N</b> <i>μg g<sup>-1</sup></i>	1.30 (± 0.12)	2.00 (± 0.35)	1.57 (± 0.28)	1.81 (± 0.25)
<b>* Biomass<sub>mic</sub></b>	5.51 (± 0.11)	7.28 (± 0.23)	6.16 (± 0.26)	5.08 (± 0.18)
<b>α-amino-N</b> <i>μg g<sup>-1</sup></i>	0.76 (± 0.06)	0.95 (± 0.05)	0.93 (± 0.06)	0.50 (± 0.11)
<b>pH</b>	8.17 (± 0.02)	8.15 (± 0.01)	8.12 (± 0.01)	8.21 (± 0.00)

\* μg α-amino-N g<sup>-1</sup>

With the exception of the CO<sub>2</sub> flux, all the parameters measured in the wheat field gave much lower values than those measured in the woodland soil (Table 3.3 and Table 3.4). A much lower biomass was present in the wheat field (about ¼ of the woodland biomass), and the content of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> were extremely low too. A much lower content of organic matter in the wheat field (see chapter 2, Table 2.1) probably supported a lower biomass than in the woodland where, instead, a constant litter layer covered the soil and provided soil micro-organisms with organic substrates and mineral nutrients. The difference of organic matter content between the arable and the woodland soil, especially in the first few cm, can probably explain also the higher pH (P<0.05) found in the top 10 cm of the wheat field soil as compared with the woodland (see chapter 2, Table 2.1).

Fluxes of N<sub>2</sub>O in the wheat field were extremely low (Figure 3.16, Table 3.7 and 3.8), as could be expected with such a low level of mineral-N present in the soil.



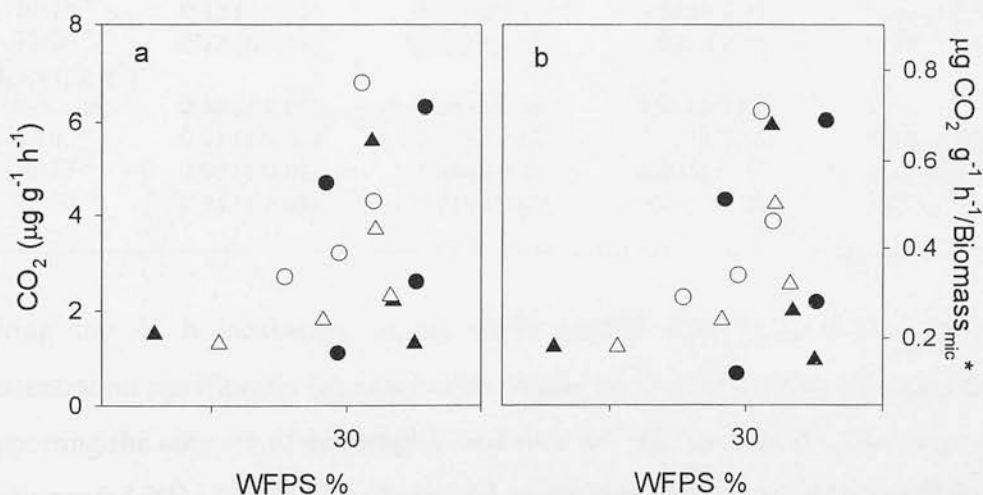
**Figure 3.16** - N<sub>2</sub>O fluxes from arable soil cores with 0%, 0.1% and 10% C<sub>2</sub>H<sub>2</sub>.

The addition of 0.1% and 10% C<sub>2</sub>H<sub>2</sub> were not really effective in discriminating between N<sub>2</sub>O sources, as the fluxes were so low (see Table II.3, *Appendix II*, for N<sub>2</sub>O fluxes in presence of C<sub>2</sub>H<sub>2</sub> in the loose soil samples).

The only factor which seemed to be correlated with the N<sub>2</sub>O flux was the microbial biomass ( $R^2 = 0.444$ ,  $P < 0.005$ ).

Soil respiration was comparable with respiration rates in the woodland and was strongly correlated with the microbial biomass ( $R^2 = 0.627$ ,  $P < 0.0005$ ).

As found for the woodland soil, CO<sub>2</sub> flux was found to increase with increasing WFPS% (Figure 3.17a, b), however the influence of depth on the CO<sub>2</sub> fluxes was much less evident than for the woodland soil (Fig. 3.15a). Also values were much more scattered than in the woodland soil showing a much weaker relationship.



**Figure 3.17** - Relation between the WFPS % and (a) the CO<sub>2</sub> flux ( $\mu\text{g g}^{-1} \text{h}^{-1}$ ) and (b) the ratio CO<sub>2</sub> flux to microbial biomass ( $\mu\text{g } \alpha\text{-amino-N g}^{-1}$ ), measured in the arable soil cores at different depths (● 0-5 cm, ○ 5-10 cm, ▲ 10-15 cm, △ 15-20 cm).

Also microbial biomass was significantly positively correlated with WFPS% ( $R^2=0.353$ ).

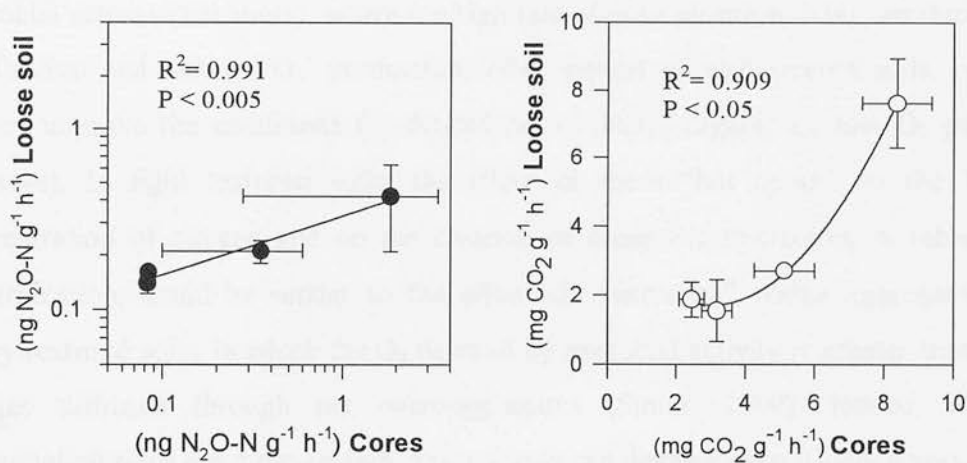
While none of the measured parameters was correlated with  $\text{NO}_3^-$ , the variations of  $\alpha$ -amino-N content of the soil explained about the 37.3% ( $P<0.05$ ) of the  $\text{NH}_4^+$  variability. The addition of  $\text{C}_2\text{H}_2$ , either 0.1% or 10%, blocked  $\text{NO}_3^-$  production at the time zero concentration (Table 3.9), suggesting that all  $\text{NO}_3^-$  was coming from autotrophic production.

**Table 3.9** - Values of  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N extracted from the wheat field soil cores after 48 h incubation in presence of 0%, 0.1% and 10%  $\text{C}_2\text{H}_2$ . In brackets is reported one standard error.

	Concentration of $\text{NH}_4^+$ and $\text{NO}_3^-$ ( $\mu\text{g N g}^{-1}$ soil)			
	<i>Initial value(<math>T_0</math>)</i>	<i>0 % <math>\text{C}_2\text{H}_2</math></i>	<i>0.1% <math>\text{C}_2\text{H}_2</math></i>	<i>10% <math>\text{C}_2\text{H}_2</math></i>
<b><math>\text{NH}_4^+</math>-N (<math>\mu\text{g g}^{-1}</math>)</b>				
0-5 cm	0.36 ( $\pm 0.04$ )	0.38 ( $\pm 0.09$ )	0.59 ( $\pm 0.04$ )	0.23 ( $\pm 0.11$ )
5-10 "	0.40 ( $\pm 0.04$ )	0.46 ( $\pm 0.12$ )	0.67 ( $\pm 0.09$ )	0.25 ( $\pm 0.03$ )
10-15 "	0.43 ( $\pm 0.02$ )	0.51 ( $\pm 0.11$ )	0.71 ( $\pm 0.14$ )	0.39 ( $\pm 0.06$ )
15-20 "	0.32 ( $\pm 0.03$ )	0.41 ( $\pm 0.15$ )	1.09 ( $\pm 0.72$ )	0.37 ( $\pm 0.03$ )
<b><math>\text{NO}_3^-</math>-N (<math>\mu\text{g g}^{-1}</math>)</b>				
0-5 cm	0.52 ( $\pm 0.03$ )	1.29 ( $\pm 0.25$ )	0.55 ( $\pm 0.02$ )	0.65 ( $\pm 0.14$ )
5-10 "	0.88 ( $\pm 0.06$ )	2.27 ( $\pm 0.18$ )	1.31 ( $\pm 0.54$ )	0.56 ( $\pm 0.34$ )
10-15 "	0.92 ( $\pm 0.06$ )	2.49 ( $\pm 0.23$ )	0.86 ( $\pm 0.12$ )	0.65 ( $\pm 0.14$ )
15-20 "	0.94 ( $\pm 0.03$ )	2.71 ( $\pm 0.47$ )	0.94 ( $\pm 0.08$ )	1.02 ( $\pm 0.24$ )

During the 48 h incubation, in the cores treated with 0.1%  $\text{C}_2\text{H}_2$ , the  $\text{NH}_4^+$  concentration significantly increased with respect to time zero concentration, further supporting the concept of autotrophic oxidation of  $\text{NH}_4^+$  as a mechanism to produce the measured  $\text{NO}_3^-$  (Table 3.9). A general condition of low organic matter input and the low water content due to the texture of the soil could have probably favoured autotrophic activity more than any heterotrophic mechanism of  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  production. Hutchinson *et al.* (1993) found that chemoautotrophic oxidizers were the predominant source of gaseous N at water contents  $<10\%$  in a sandy loam, furthermore the addition of nitrapyrin (a nitrification inhibitor) blocked the process.

In conclusion, only the woodland soil showed significant N<sub>2</sub>O production. While N<sub>2</sub>O production from cores was about 4 times higher than in the loose samples, CO<sub>2</sub> production rates were comparable (Figure 3.18). Consequently the difference in N<sub>2</sub>O production could not be attributed to a different microbial growth and respiratory consumption of O<sub>2</sub>.



**Figure 3.18** - Correlation between fluxes of N<sub>2</sub>O and CO<sub>2</sub> in the woodland soil cores and loose soil samples.

As described before (Table 3.5), the highest N<sub>2</sub>O fluxes from soil cores were associated with high levels of NH<sub>4</sub><sup>+</sup> and fresh organic matter. Bergstrom *et al.* (1994) observed that soil cores, collected from a grassland, containing high NH<sub>4</sub><sup>+</sup> and C substrate concentrations, produced relatively large quantities of N<sub>2</sub>O as compared with samples receiving only NH<sub>4</sub><sup>+</sup> or nothing, in aerobic conditions, and that addition of either NH<sub>4</sub><sup>+</sup> or NH<sub>4</sub><sup>+</sup> plus glucose, induced the same rate of CO<sub>2</sub> production, suggesting, as in the present experiment, that the overall respiration activity was not directly related to the N<sub>2</sub>O flux differences. Furthermore they noticed that in the presence of C<sub>2</sub>H<sub>2</sub> the N<sub>2</sub>O emissions were enhanced and they supposed, consequently, that denitrification could have been the source of those emissions. However, repeating



the experience on anaerobic soil slurries, they found that  $\text{NH}_4^+$  did not directly affect  $\text{N}_2\text{O}$  production by denitrification.

Three different sources of  $\text{N}_2\text{O}$  could be hypothesised on the basis of the results presented here. The first is represented by denitrification activity, which could be enhanced in presence of  $\text{NH}_4^+$  and organic matter. In aerobic soils, the fresh organic matter present could eventually cause the development of microsites of intense microbial activity (hot spots), where the high rate of mineralization,  $\text{NH}_4^+$  production, nitrification and hence  $\text{NO}_3^-$  production, often typical of well-aerated soils, could further improve the conditions for denitrification ( $\text{NO}_3^-$ , organic C, low  $\text{O}_2$  partial pressure). In light textured soils, the effect of these “hot spots” on the local concentration of oxygen and on the creation of anaerobic microsites, suitable for denitrification, could be similar to the effect of “microsites” within aggregates of heavy textured soils, in which the  $\text{O}_2$  demand by microbial activity is greater than the oxygen diffusion through the microaggregates (Smith, 1990). Indeed, if  $\text{O}_2$  consumption rates are great enough, anaerobiosis can develop even if only a very thin film of water is limiting  $\text{O}_2$  diffusion (Parkin, 1987).

The other two sources of  $\text{N}_2\text{O}$  could be represented by autotrophic and heterotrophic nitrification activity. As described in § 1.4,  $\text{N}_2\text{O}$  production via nitrification is also increased by reduced  $\text{O}_2$  partial pressure as  $\text{NO}_2^-$  is used instead of  $\text{O}_2$  for processes of respiration. The fresh organic matter in soil could favour the process by reducing  $\text{O}_2$  partial pressure, it could be a source of  $\text{NH}_4^+$  via mineralization and could be a C substrate for heterotrophic nitrification. In this case no  $\text{NO}_3^-$  would be needed to have high  $\text{N}_2\text{O}$  fluxes. It is possible that all the three processes could happen contemporaneously in the soil, though their relative importance could change with the variation of the environmental factors.

In the arable soil cores the levels of mineral N and the available fresh organic matter were extremely low and this was probably the reason for extremely low  $\text{N}_2\text{O}$  fluxes. In such soil, “hot spots” of microbial activity leading to production of  $\text{N}_2\text{O}$ ,  $\text{CO}_2$ ,

$\text{NH}_4^+$ , etc., could have been even more random than in the woodland and the possibilities for creating optimal conditions for high  $\text{N}_2\text{O}$  fluxes were probably much lower. In such circumstances it is not surprising that no correlation was found, for  $\text{CO}_2$  or  $\text{N}_2\text{O}$  fluxes, between soil cores and loose soil samples.

### 3.4 CONCLUSIONS

Microbial processes and the soil properties, which are influenced by microbial activity, typically display high variability, due to the dynamic nature of such processes, often showing a highly skewed frequency distribution (Parkin, 1987). Multiplicative effects of environmental factors have been proposed as the source of such a distribution (Aitchinson and Brown, 1957) together with the high spatial variability of soil properties (Folorunso and Rolston, 1984; Parkin, 1987).

Soil cores results showed high  $\text{N}_2\text{O}$  emissions rates associated with “hot spots” of microbial activity, characterised by presence of fresh organic matter and high concentrations of  $\text{NH}_4^+$ . In the field the dispersion of those hot spots can vary temporally and also spatially in response to changing conditions of the soil. Indeed, when  $(\text{NH}_4)_2\text{SO}_4$  was added to the woodland soil (exp. II), the treated plot showed higher  $\text{N}_2\text{O}$  emissions overall but also higher variability of the emission rates, than the control plot. This suggests the presence of different sources of  $\text{N}_2\text{O}$ : a first source which was homogeneous and was giving a constant higher flux in the treated plot than in the control plot and a second source or process which had a non-homogeneous distribution and was responsible for the higher and rarer peaks. To identify the latter source of  $\text{N}_2\text{O}$ , a high frequency of sampling was needed, as evidenced before in this chapter.

The low emissions of  $\text{N}_2\text{O}$  measured throughout one year in the woodland soil and in the wheat field could be an underestimation of the flux due to the frequency of sampling. The wheat soil, however, showed very low  $\text{N}_2\text{O}$  emission rates also in the core incubation, which can be explained more as a lack of optimal condition for  $\text{N}_2\text{O}$

emissions (low content of fresh organic matter, low water content, almost no structure in the soil) than as a lower microbial activity overall. Indeed, even though the arable soil microbial biomass was much lower than in the woodland, it exhibited respiration rates comparable to those in the woodland, indicating a very active microflora.

The woodland soil exhibited a high nitrifying activity, producing high levels of  $\text{NO}_3^-$  when fertilizer was added as  $\text{NH}_4^+\text{-N}$  to the soil. A lower  $\text{NO}_3^-$  production was measured, instead, in the arable soil. It is not clear if the nitrification process was the source of  $\text{N}_2\text{O}$  in the field, as no direct correlation was found between mineral N (in particular  $\text{NO}_3^-$ ) and  $\text{N}_2\text{O}$  emissions. Moreover, it was also not clear which was the main process responsible for the high  $\text{N}_2\text{O}$  emissions rates from hot spots, as when the woodland soil was fertilized or in soil cores, the higher emissions were associated with high concentrations of  $\text{NH}_4^+$  rather than high concentration of  $\text{NO}_3^-$ . Other authors have found this correlation between high  $\text{N}_2\text{O}$  fluxes and high  $\text{NH}_4^+$  content, suggesting that  $\text{NH}_4^+$  concentration, in addition to  $\text{NO}_3^-$ , C and  $\text{O}_2$  concentrations, is an important factor in predicting  $\text{N}_2\text{O}$  production by aerobic and anaerobic processes in the field (Peterjohn, 1991; Ambus *et al.*, 1993; Colbourn, 1993; Bergstrom *et al.*, 1994). Robertson and Tiedje (1987) have found similar rates of  $\text{N}_2\text{O}$  production in soil cores from two loamy sand forest soils, and considered nitrifiers and denitrifiers both important sources of  $\text{N}_2\text{O}$ . They also proposed the possibility for an alternative source of  $\text{N}_2\text{O}$  which was inhibited neither by  $\text{C}_2\text{H}_2$  or  $\text{O}_2$ .

Different possible sources for such emissions have been hypothesised in the discussion: i) denitrification, ii) autotrophic nitrification and iii) heterotrophic nitrification.

In the following laboratory experimental part of this thesis, possible biological sources of  $\text{N}_2\text{O}$  were investigated in the studied soils.

## MICROBIAL SOURCES OF N<sub>2</sub>O IN THE WOODLAND AND IN THE ARABLE SOIL

### 4.1 BIOLOGICAL N<sub>2</sub>O PRODUCTION FROM DIFFERENT N SOURCES IN THE WOODLAND AND IN THE ARABLE SOIL<sup>(1)</sup>

#### 4.1.1 INTRODUCTION

Different forms of N can have very different effects on soil N transformations and N<sub>2</sub>O emissions, as they can be differently and/or selectively used by specific kinds of soil microorganisms.

Ammonium and ammonium-producing compounds are the most widely used forms of N fertiliser applied to soil. It is generally recognised that autotrophic nitrification by organisms such as *Nitrosomonas*, *Nitrosolobus* and *Nitrospira*, is the main process involved in ammonium oxidation and N<sub>2</sub>O production in well-aerated soils (Blackmer *et al.*, 1980; Tortoso and Hutchinson, 1990; Davidson, 1992). Blackmer *et al.* (1980) found that N<sub>2</sub>O emissions were much higher in a soil treated with nitrifiable N than in plots treated with calcium nitrate, even when the soil samples were saturated with <sup>1</sup>water. N<sub>2</sub>O emissions deriving from the ammonium treated plots were also found to increase with increasing water content; this was in accord with Ritchie and Nicholas (1972), who suggested that *Nitrosomonas europea* can utilise the nitrite produced

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<sup>1</sup> A paper entitled "The effect of different N substrates on biological N<sub>2</sub>O production from forest and agricultural light textured soils" based on the results presented in this paragraph has been accepted for publication in December 1997 on *Plant and Soil*.

from ammonium oxidation as a terminal electron acceptor, with production of  $\text{N}_2\text{O}$ , when the supply of oxygen is not sufficient for its requirements. Indeed, many authors found that nitrifiers are able to produce  $\text{N}_2\text{O}$  from nitrite; however, ammonium was also required in the culture medium or in the soil for significant nitrite reduction and  $\text{N}_2\text{O}$  production to occur (Blackmer *et al.*, 1980; Poth and Focht, 1985; Abeliovich and Vonshak, 1992).

As discussed in § 1.4.2, many soil microorganisms have been found to be able to produce  $\text{N}_2\text{O}$  from denitrification of nitrate and nitrite over a wide range of oxygen partial pressures (Lloyd *et al.*, 1987; Davies *et al.*, 1989; Robertson and Kuenen, 1990).

Heterotrophic nitrifiers have been found to be able to produce  $\text{N}_2\text{O}$  by utilising more complex N substrates such as aminoacids or peptone (Van Gool and Schmidt, 1973; Van de Dijk and Troelstra, 1980; Schimel *et al.*, 1984, Papen *et al.*, 1989).

In the following experiment, the effect of addition of different N substrates on  $\text{N}_2\text{O}$  emissions, from the woodland and the arable soil, was evaluated. Where the added nitrogen ( $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) was to be tested as an electron acceptor for heterotrophic catabolic microbial processes, glucose was added as well. Peptone was used as a more complex form of N to stimulate specifically heterotrophic activity.

As nitrite has been reported to be implicated in several non-biological reactions with  $\text{NH}_4^+$  (Allison, 1965), with clay (Bulla *et al.*, 1970), and with soil organic matter (Bremner and Fuhr, 1963), all of which lead in general to the release of gaseous forms of nitrogen, the effect of  $\text{NO}_2^-$  addition on  $\text{N}_2\text{O}$  emissions was also tested on the soil which had been previously sterilised. Due to the characteristics of the two soils (described previously in chapter 3), only the woodland soil was tested as it was expected to be potentially a more suitable environment for such reactions than the arable soil.

#### 4.1.2 EXPERIMENTAL DESIGN

For each soil, five sub-samples were taken randomly from the top 10 cm and mixed in plastic bags. Soil was sieved (2 mm) the same day and stored at room temperature ( $\sim 20^{\circ}\text{C}$ ).

In the first experiment, the soils were mixed with substrates, added as powder (talcum 0.5 g 100 g<sup>-1</sup> soil was used as an inert carrier medium). The control received only talcum. The added substrate were (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (AS), NaNO<sub>2</sub> (SN), NaNO<sub>2</sub> plus glucose (SN+G), Ca(NO<sub>3</sub>)<sub>2</sub> plus glucose (CN+G) and peptone (P). NaNO<sub>2</sub> and Ca(NO<sub>3</sub>)<sub>2</sub> were readily available in the laboratory, and before starting the experiments no significant evidence was found in the literature of different behaviour of Ca and Na salts in soil denitrification, at these concentrations. N additions were 70  $\mu\text{g N g}^{-1}$  dry soil. In a preliminary experiment such a quantity was shown to be sufficiently high to give a detectable increase in N<sub>2</sub>O-N in 24 h incubation. Moreover, it was also in the range of typical N amendments to soil. Glucose was added at 300  $\mu\text{g C g}^{-1}$  dry soil (C/N 4.3). A C/N ratio of 3-4/1 has been found optimum when glucose is used as a electron donor in the denitrification process (Bremner and Shaw, 1958; Thomas *et al.*, 1994). The water content was adjusted to 60% WFPS (see § 2.7.6). Soil samples (three replicates per each substrate) of 50 g were incubated in 1 l air-tight jars (see § 2.3). Immediately after the lids were closed, three replicates for each treatment received 1 ml l<sup>-1</sup> C<sub>2</sub>H<sub>2</sub> (0.1% or 100Pa C<sub>2</sub>H<sub>2</sub>) to block the activity of ammonia-monooxygenase (Hynes and Knowles, 1978) and three replicates received 100 ml l<sup>-1</sup> C<sub>2</sub>H<sub>2</sub> (10% or 10kPa C<sub>2</sub>H<sub>2</sub>) to block nitrous oxide reductase activity (Yoshinari and Knowles, 1976), in order to distinguish N<sub>2</sub>O produced by autotrophic nitrification from N<sub>2</sub>O derived from the denitrification (or heterotrophic nitrification) and N<sub>2</sub> produced by denitrification (Davidson *et al.*, 1986) (see § 2.5 for more details on the acetylene block technique). When 10% C<sub>2</sub>H<sub>2</sub> was added, 100 ml of air were first removed from the jar using a syringe.



Soil was incubated at  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$  in a thermostated room. After 24 h gas samples were taken with 2 ml glass syringes and analysed for  $\text{N}_2\text{O}$ .  $\text{O}_2$  concentration in the head space was not measured during the present experiment as in a preliminary experiment, where both soils had been incubated under the same conditions, the  $\text{O}_2$  concentration in the headspace of the incubation jars had been followed for 7 days and did not fall significantly below ambient concentration in the first 48 h. As the soil represented only 5% of the total internal volume of the jar, and had been sieved and was kept at 60% WFPS, it is reasonable to assume that the environment was mainly aerobic

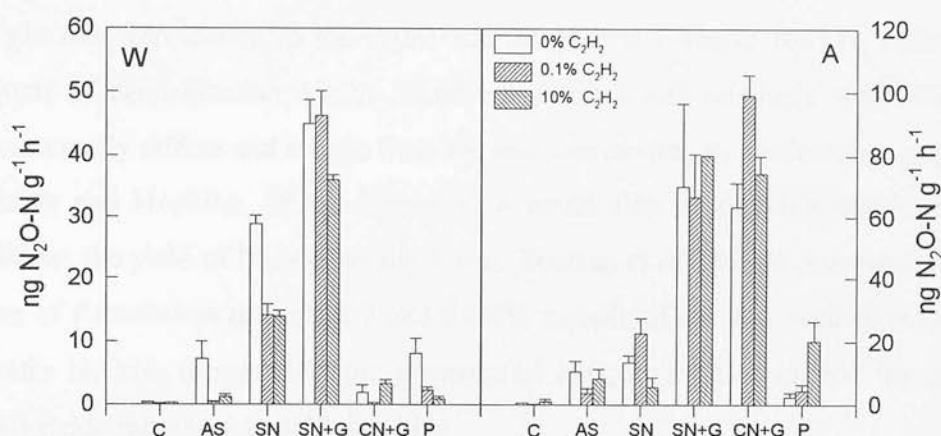
Sub-samples of soil, from each jar, were extracted for mineral N. Mineral N in the bulk soil was also measured at time zero.

In a second experiment, the woodland soil was first autoclaved at  $121^{\circ}\text{C}$  (autoclave pressure at 103.35 kPa) for 30 min. It was then left to dry in the oven at  $110^{\circ}\text{C}$  for a few hours. The water content was then measured. The next day both the non-autoclaved and the autoclaved soils were prepared for incubation. Soil was treated with no substrate (control), with  $\text{NaNO}_2$  ( $70\ \mu\text{g N g}^{-1}$  soil), and with  $\text{NaNO}_2$  ( $70\ \mu\text{g N g}^{-1}$  soil) plus glucose ( $300\ \mu\text{g C g}^{-1}$  soil). Talcum was used as a carrier medium for the substrates. The water content of the soil was brought to 60% of WFPS. For each treatment 3 replicates, of 50 g each, were incubated in 1 l air-tight jars with zero, 0.1 % and 10 %  $\text{C}_2\text{H}_2$  (for a total of 9 replicates per each treatment). Samples were incubated at  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . After 24 h gas samples were taken for  $\text{N}_2\text{O}$  analysis and soil was extracted for mineral N analysis, as in the previous experiment.

#### *4.1.3 RESULTS AND DISCUSSION*

##### Effects of different N sources on $\text{N}_2\text{O}$ production and mineral N

The response of  $\text{N}_2\text{O}$  fluxes to the addition of different forms of N substrate was quite different in the two soils (Figure 4.1).



**Figure 4.1** - Emission rates of N<sub>2</sub>O from the woodland (W) and the arable (A) soil amended with different substrates.

In the control N<sub>2</sub>O flux was very low, while (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> addition increased N<sub>2</sub>O emissions up to 8-10 ng N<sub>2</sub>O-N g<sup>-1</sup> h<sup>-1</sup> in both soils. 0.1% C<sub>2</sub>H<sub>2</sub> or 10% C<sub>2</sub>H<sub>2</sub> significantly reduced these N<sub>2</sub>O emissions, particularly in the woodland soil.

When NaNO<sub>2</sub> was added to the woodland soil the N<sub>2</sub>O flux significantly increased, reaching 28.8 ng N g<sup>-1</sup> h<sup>-1</sup>, and both 0.1% or 10% C<sub>2</sub>H<sub>2</sub> reduced N<sub>2</sub>O emissions by half. In the arable soil, the effect of NaNO<sub>2</sub> addition was comparable to that of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, except that 0.1% C<sub>2</sub>H<sub>2</sub> did not reduce the N<sub>2</sub>O flux. When NaNO<sub>2</sub> was added together with glucose, the N<sub>2</sub>O fluxes substantially increased, and were not significantly reduced by C<sub>2</sub>H<sub>2</sub> addition in either soils.

The addition of Ca(NO<sub>3</sub>)<sub>2</sub> plus glucose had very different effects on the two soils. Whereas in the woodland soil N<sub>2</sub>O emissions were extremely low (1.8 ng N<sub>2</sub>O-N g<sup>-1</sup> h<sup>-1</sup>), in the arable soil they reached 63.3 ng N<sub>2</sub>O-N g<sup>-1</sup> h<sup>-1</sup>, which was comparable to the increase in N<sub>2</sub>O emissions measured when NaNO<sub>2</sub> plus glucose were added. Addition of 0.1% C<sub>2</sub>H<sub>2</sub> to the woodland soil generally caused a significant reduction in N<sub>2</sub>O emissions, indicating that the source of such emissions was not the denitrification of the added NO<sub>3</sub><sup>-</sup>. In contrast, in the arable soil, 0.1% C<sub>2</sub>H<sub>2</sub> did not

reduce  $\text{N}_2\text{O}$  emissions. However, 10%  $\text{C}_2\text{H}_2$  (inhibitor of the nitrous oxide reductase) did not enhance  $\text{N}_2\text{O}$  emissions, either when  $\text{NaNO}_2$  plus glucose or when  $\text{Ca}(\text{NO}_3)_2$  plus glucose were added to the arable soil. In general a bigger  $\text{N}_2\text{O}:\text{N}_2$  ratio in the products of denitrification can be found when soil is still relatively well-aerated as  $\text{N}_2\text{O}$  can easily diffuse and escape from the sink represented by denitrifying organisms (Webster and Hopkins, 1996). However, it could also be possible that in aerobic conditions the yield of  $\text{N}_2$  is generally lower. Thomas *et al.* (1994) observed, for two strains of *Pseudomonas* (isolate 7 and PAO1) capable of aerobic denitrification, that the ratio  $\text{N}_2\text{O}:\text{N}_2$  increased in the presence of oxygen, suggesting that the enzyme nitrous oxide reductase was  $\text{O}_2$ -sensitive.

$\text{N}_2\text{O}$  emissions stimulated by peptone addition were significantly higher in the woodland than in the arable soil. The effect of  $\text{C}_2\text{H}_2$  was very different in the two soils, as 0.1% or 10%  $\text{C}_2\text{H}_2$  reduced drastically such emissions in the woodland soil, while they were ineffective in reducing  $\text{N}_2\text{O}$  emissions in the arable soil.

Values of  $\text{NH}_4^+\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  measured after 24 h in the samples are reported in Table 4.1;  $\text{NO}_2^-\text{-N}$  is not reported as measured values were always  $<1 \mu\text{g N g}^{-1}$ .

$\text{NH}_4^+\text{-N}$  was extremely low for all the treatments with zero  $\text{C}_2\text{H}_2$ , except in the arable soil treated with  $(\text{NH}_4)_2\text{SO}_4$ , where after 24 h about half of the added  $\text{NH}_4^+\text{-N}$  was still present in the soil. When 0.1% or 10%  $\text{C}_2\text{H}_2$  were added, a significant increase of  $\text{NH}_4^+$  was measured for almost all the treatments. The biggest increase was measured in the  $(\text{NH}_4)_2\text{SO}_4$  treated samples, indicating that most of the  $\text{NH}_4^+$ , which had disappeared in the absence of  $\text{C}_2\text{H}_2$ , had been nitrified.

In the woodland soil treated with  $(\text{NH}_4)_2\text{SO}_4$ , the  $\text{NH}_4^+$  which accumulated in the presence of  $\text{C}_2\text{H}_2$  (about  $52\text{-}55 \mu\text{g N g}^{-1} \text{ d}^{-1}$ ) was comparable to the quantity of  $\text{NO}_3^-$  produced in the absence of  $\text{C}_2\text{H}_2$ . This  $\text{NO}_3^-$  corresponds to the total  $\text{NO}_3^-$  measured with zero  $\text{C}_2\text{H}_2$  ( $71.7 \mu\text{g N g}^{-1}$ ) minus the  $\text{NO}_3^-$  found in the soil (about  $10 \mu\text{g N g}^{-1}$ ) in presence of  $\text{C}_2\text{H}_2$ . The latter could have been derived from some other process, such as heterotrophic nitrification.

**Table 4.1** - Average values of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$ , measured in the woodland and arable soil samples after 24 hours. (In brackets is reported one standard error).

Treatments	$\text{NH}_4^+\text{-N}$ ( $\mu\text{g N g}^{-1}$ )			$\text{NO}_3^-\text{-N}$ ( $\mu\text{g N g}^{-1}$ )		
	Acetylene			Acetylene		
	0%	0.1%	10%	0%	0.1%	10%
<i>Woodland</i>						
C	1.0 (0.1)	4.4 (0.1)	5.1 (0.7)	8.0 (0.0)	7.9 (0.9)	7.0 (1.0)
AS	1.1 (0.1)	55.4 (2.3)	52.7 (0.9)	71.7 (2.4)	13.8 (1.0)	6.9 (0.1)
SN	1.7 (0.2)	16.6 (0.3)	16.8 (0.9)	81.7 (2.5)	63.6 (0.4)	64.2 (2.0)
SN + G	0.6 (0.0)	10.2 (0.3)	11.9 (0.0)	76.7 (1.5)	60.1 (1.2)	62.0 (5.5)
CN + G	0.5 (0.0)	0.7 (0.0)	0.6 (0.0)	59.5 (1.0)	71.3 (7.0)	69.7 (7.2)
P	1.2 (0.0)	22.4 (0.1)	17.7 (0.3)	48.2 (2.2)	9.4 (1.0)	7.9 (0.6)
<i>Wheat field</i>						
C	0.3 (0.0)	0.8 (0.3)	1.7 (0.1)	3.5 (0.0)	3.1 (0.0)	3.0 (0.1)
AS	26.3 (9.7)	47.4 (2.3)	52.4 (1.4)	6.7 (3.4)	9.9 (2.6)	1.5 (0.2)
SN	0.8 (0.0)	5.8 (0.2)	4.8 (0.0)	60.9 (2.1)	53.5 (2.9)	53.8 (2.8)
SN + G	3.9 (1.4)	5.5 (0.3)	3.5 (0.1)	36.0 (4.5)	49.0 (2.2)	34.7 (2.9)
CN + G	0.3 (0.3)	1.1 (0.3)	0.2 (0.1)	36.8 (9.5)	44.1 (8.5)	36.7 (ND)
P	0.3 (0.0)	21.2 (0.1)	18.4 (0.4)	7.5 (3.1)	4.7 (0.6)	2.0 (1.8)

In the arable soil the nitrifier population seemed much less effective in oxidising  $\text{NH}_4^+\text{-N}$  to  $\text{NO}_3^-\text{-N}$  as a very low content of  $\text{NO}_3^-$  was found in the soil, either with or without  $\text{C}_2\text{H}_2$ . However,  $\text{NH}_4^+$  accumulation in the soil in presence of  $\text{C}_2\text{H}_2$  indicated that about  $20 \mu\text{g NH}_4^+\text{-N g}^{-1}$  were nitrified. It is improbable that part of the  $\text{NO}_3^-$  formed could have been immobilised and consequently not recovered as available  $\text{NO}_3^-$ , as it has been shown that when  $\text{NH}_4^+$  is present together with  $\text{NO}_3^-$ , even at very low concentrations, it blocks  $\text{NO}_3^-$  uptake and immobilisation by microorganisms (Rice and Tiedje, 1989). In our case, after 24 h, there were still  $26.3 \mu\text{g of NH}_4^+\text{-N g}^{-1}$  which had not been immobilised, thus reducing the probability that the  $\text{NO}_3^-$  present could have been immobilised by soil microflora. It is possible that the  $\text{NO}_3^-$  produced by nitrification of the added  $(\text{NH}_4)_2\text{SO}_4$  could have been immediately reduced (by aerobic denitrifiers), so that the net oxidation of  $(\text{NH}_4)_2\text{SO}_4$  was only apparently low.

A similar situation was found in the arable soil when peptone was added. Whereas in the woodland soil considerable  $\text{NO}_3^-$  was recovered ( $48.2 \mu\text{g N g}^{-1} \text{d}^{-1}$ ), in the arable soil the  $\text{NO}_3^-$  found after 24 h was very low ( $7.5 \mu\text{g N g}^{-1} \text{d}^{-1}$ ). However, a significant quantity of  $\text{NH}_4^+$  accumulated in both soils treated with peptone and  $\text{C}_2\text{H}_2$ , which suggested that in both soils the mineralization activity was high and was of the same order of magnitude and that in both soils  $\text{NH}_4^+$  was being nitrified via a pathway involving ammonia-monooxygenase.

Almost no  $\text{NO}_2^-$ -N was recovered in both soils after 24 h incubation, even where  $\text{NaNO}_2$  had been added. This indicated that soil microorganisms were able to oxidise  $\text{NO}_2^-$  very efficiently. Most of the added  $\text{NO}_2^-$  was oxidised to  $\text{NO}_3^-$  in both soils (Table 4.1). Addition of  $\text{C}_2\text{H}_2$  only slightly reduced  $\text{NO}_3^-$  production (as  $\text{C}_2\text{H}_2$  only blocks the oxidation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$ ), probably blocking the basal  $\text{NO}_3^-$  production of the soil (Table 4.1).

From the  $\text{N}_2\text{O}$  and mineral N data, it seems that more than one mechanism was involved in  $\text{N}_2\text{O}$  emissions and that those mechanisms were of different importance in the two soils.

Autotrophic nitrification seemed to make a significant contribution to  $\text{N}_2\text{O}$  fluxes in both soils; however, the nitrifier population seemed, apparently, significantly less efficient in the arable land than in the woodland (Table 4.2). The population of nitrite oxidisers, instead, was equally efficient in both soils (Tables 4.1 and 4.2).

The dramatic increase in  $\text{N}_2\text{O}$  fluxes measured in the arable soil, when  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were added together with glucose, suggested that an active population of aerobic denitrifiers was present, which was yielding, however, mainly  $\text{N}_2\text{O}$  and not  $\text{N}_2$ . The yield of  $\text{N}_2\text{O}$  did not account, however, for all consumed mineral N. It is possible that significant quantities of NO could also have been produced. It is reported that generally the ratio  $\text{NO}:\text{N}_2\text{O}$  is very high in aerobic soil (Skiba *et al.*, 1992). Denitrifiers could have been responsible for the small  $\text{N}_2\text{O}$  flux measured when  $\text{NO}_2^-$

was added without glucose, as the flux was not significantly affected by  $C_2H_2$ . C substrates already present in the soil could have been sufficient to allow for  $NO_3^-$  reduction. An active population of denitrifiers could also explain the low concentration of  $NO_3^-$  found in soil, despite a significant level of nitrification evidenced by  $NH_4^+$  accumulation in presence of  $C_2H_2$ , when  $(NH_4)_2SO_4$  or peptone were added.

**Table 4.2** - Net rates of  $NO_3^-$ -N ( $\mu g\ N\ g^{-1}\ h^{-1}$ ) production (+) or consumption (-) in the woodland and in the arable soil treated with different substrates and incubated with 0%, 0.1% and 10%  $C_2H_2$ .

Treatments	$NO_3^-$ production or consumption rate ( $\mu g\ N\ g^{-1}\ h^{-1}$ )		
	0% $C_2H_2$	0.1% $C_2H_2$	10% $C_2H_2$
<i>Woodland soil</i>			
C	+ 0.21 <sup>a4</sup>	+ 0.21 <sup>a4</sup>	+ 0.17 <sup>b3</sup>
AS	+ 2.53 <sup>a2</sup>	+ 0.12 <sup>b2</sup>	- 0.16 <sup>c2</sup>
SN	+ 2.95 <sup>a1</sup>	+ 2.19 <sup>b1</sup>	+ 2.26 <sup>b1</sup>
SN+G	+ 2.74 <sup>a1</sup>	+ 2.05 <sup>b1</sup>	+ 2.17 <sup>b1</sup>
CN+G	- 0.98 <sup>a5</sup>	- 0.48 <sup>a5</sup>	- 0.47 <sup>a4</sup>
P	+ 1.55 <sup>a3</sup>	- 0.06 <sup>b3</sup>	- 0.08 <sup>b2</sup>
<i>Arable soil</i>			
C	+ 0.02 <sup>a3</sup>	+ 0.003 <sup>b3</sup>	- 0.002 <sup>b3</sup>
AS	+ 0.02 <sup>a3</sup>	+ 0.16 <sup>a2</sup>	- 0.18 <sup>b4</sup>
SN	+ 2.26 <sup>a1</sup>	+ 1.97 <sup>b1</sup>	+ 1.99 <sup>b1</sup>
SN+G	+ 1.23 <sup>a2</sup>	+ 1.79 <sup>b1</sup>	+ 1.20 <sup>a2</sup>
CN+G	- 1.55 <sup>a2</sup>	- 1.21 <sup>a5</sup>	- 1.51 <sup>a5</sup>
P	+ 0.04 <sup>a3</sup>	- 0.06 <sup>ab4</sup>	- 0.16 <sup>b4</sup>

N.B. Different letters and numbers in superscript indicate significant differences in columns and rows, respectively.

In the woodland, the extremely low  $N_2O$  emissions and denitrification rates induced by  $Ca(NO_3)_2$  addition suggested that denitrifiers were not playing an important role. In contrast, very high  $N_2O$  fluxes were induced by  $NaNO_2$ . This suggests that, in contrast with the situation in the arable soil, the  $NO_2^-$  and  $NO_3^-$  were utilised by different processes or routes.



The  $\text{N}_2\text{O}$  flux stimulated by  $\text{NO}_2^-$  addition and inhibited by  $\text{C}_2\text{H}_2$  could derive from autotrophic nitrifiers reduction of  $\text{NO}_2^-$ . Several authors have found that such a reduction of  $\text{NO}_2^-$  to  $\text{N}_2\text{O}$  by autotrophic nitrifiers was particularly enhanced by addition of  $\text{NH}_4^+$  and electron donors (Blackmer *et al.*, 1980; Abieliovich and Vonshak, 1992). Poth and Focht (1985) concluded that the inhibition of ammonia oxidation by nitrification inhibitors would eliminate the source of electrons (hydroxylamine) for nitrite reduction and hence  $\text{N}_2\text{O}$  production. Indeed production of  $\text{N}_2\text{O}$  by such microorganisms has been found to be correlated with  $\text{NH}_4^+$  but not with  $\text{NO}_3^-$  additions. This would be in accord with the results presented for the woodland soil, where  $\text{Ca}(\text{NO}_3)_2$  addition did not stimulate any significant emission of  $\text{N}_2\text{O}$ . The  $\text{N}_2\text{O}$  flux not inhibited by  $\text{C}_2\text{H}_2$  could be due the heterotrophic nitrifiers. Indeed, when glucose was added together with  $\text{NO}_2^-$ ,  $\text{N}_2\text{O}$  emissions significantly increased. Several heterotrophic microorganisms such as *Arthrobacter* sp. and *Alcaligenes faecalis* have been found to be able to nitrify, but as the process consumed energy it was carried out when a source of energy was supplied (Castignetti, 1990). Denitrification could be a less probable source as it is not clear why glucose plus  $\text{NO}_3^-$  (which is a more oxidized form as compared with  $\text{NO}_2^-$ ) did not yield any significant quantity of  $\text{N}_2\text{O}$ .

Heterotrophic nitrifiers could also have had a major role in the  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  production stimulated by peptone addition in the woodland soil. However,  $\text{N}_2\text{O}$  emissions and  $\text{NO}_3^-$  production were almost completely blocked by  $\text{C}_2\text{H}_2$  addition. Fungi and heterotrophic bacteria are generally believed to nitrify through some organic pathway (Verstraete and Alexander, 1972; Killham, 1986). However, Papen *et al.* (1989) have found that heterotrophic nitrification by *Alcaligenes faecalis* was not restricted to media containing nitrogen in organic form but could also have been achieved with ammonium. The presence of an enzyme very similar to the ammonia-monooxygenase has been demonstrated in the bacterium *Tsa. Pantotropha* (Robertson and Kuenen, 1990). However, more studies are needed to clarify the pathway of nitrate production by heterotrophic microorganisms.

Addition of NaNO<sub>2</sub>, with or without glucose, to the autoclaved soil induced a slight increase in the N<sub>2</sub>O emissions, which, though significantly higher than the control, was very small compared with the fluxes observed in the non-autoclaved soil (Table 4.3).

**Table 4.3** - Fluxes of N<sub>2</sub>O measured in the autoclaved and not-autoclaved woodland soil untreated, or treated with NaNO<sub>2</sub> or NaNO<sub>2</sub> and glucose (G), in the presence of 0%, 0.1%, and 10% C<sub>2</sub>H<sub>2</sub>.

	Flux (ng N <sub>2</sub> O-N g <sup>-1</sup> h <sup>-1</sup> )					
	NOT-AUTOCLAVED			AUTOCLAVED		
	0 % C <sub>2</sub> H <sub>2</sub>	0.1 % C <sub>2</sub> H <sub>2</sub>	10 % C <sub>2</sub> H <sub>2</sub>	0 % C <sub>2</sub> H <sub>2</sub>	0.1 % C <sub>2</sub> H <sub>2</sub>	10 % C <sub>2</sub> H <sub>2</sub>
Untreated	0.40 ± 0.00	- 0.02 ± 0.02	- 0.06 ± 0.01	- 0.07 ± 0.02	- 0.07 ± 0.02	- 0.07 ± 0.01
NaNO <sub>2</sub>	28.07 ± 0.50	15.97 ± 3.51	11.04 ± 0.30	0.27 ± 0.04	0.23 ± 0.05	0.18 ± 0.07
NaNO <sub>2</sub> + G	45.47 ± 2.19	43.73 ± 4.83	23.10 ± 14.30	0.24 ± 0.07	0.20 ± 0.07	0.16 ± 0.04

Addition of 0.1% and 10% C<sub>2</sub>H<sub>2</sub> did not induce any significant change in the N<sub>2</sub>O emissions in the autoclaved soil. In the non-autoclaved soil the results obtained for N<sub>2</sub>O emissions in the presence of zero, 0.1% and 10% C<sub>2</sub>H<sub>2</sub> were comparable with the results obtained in the first experiment with the same treatments (Figure 4.1).

Values of mineral N in the non-autoclaved soil (Tables 4.4) were also comparable with the values obtained in the first experiment (Table 4.1).

Values of NO<sub>3</sub><sup>-</sup> in the control were slightly higher than in the first experiment. This was probably due to the longer time that passed between field sampling and the laboratory incubation as compared with the first experiment, which allowed the soil to be kept longer at a higher temperature (than in the field), probably increasing the general rate of the microbial processes. Slightly less NO<sub>3</sub><sup>-</sup> was found in the samples treated with NaNO<sub>2</sub>, but slightly more NO<sub>2</sub><sup>-</sup> was detected in the samples after 24 h.

**Table 4.4** - Average values of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$  and  $\text{NO}_3^-\text{-N}$ , measured after 24 h incubation in the autoclaved and non autoclaved woodland soil, untreated, treated with  $\text{NaNO}_2$  or with  $\text{NaNO}_2$  and glucose (G). Values in brackets represent one standard error.

Treatments	$\text{NH}_4^+\text{-N}$ ( $\mu\text{g g}^{-1}$ )			$\text{NO}_2^-\text{-N}$ ( $\mu\text{g g}^{-1}$ )			$\text{NO}_3^-\text{-N}$ ( $\mu\text{g g}^{-1}$ )		
	Acetylene			Acetylene			Acetylene		
	0%	0.1%	10%	0%	0.1%	10%	0%	0.1%	10%
<i>autoclaved</i>									
Untreated	65.6 (2.1)	65.2 (2.1)	63.3 (4.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	7.2 (0.3)	7.7 (0.2)	7.6 (0.3)
$\text{NaNO}_2$	56.5 (1.7)	56.9 (0.8)	58.6 (3.2)	61.8 (2.4)	59.9 (2.3)	61.6 (5.9)	22.2 (2.2)	26.5 (1.4)	28.3 (1.4)
$\text{NaNO}_2$ + G	55.8 (2.2)	56.5 (0.5)	53.7 (4.2)	53.5 (3.2)	52.9 (1.6)	60.1 (3.6)	29.8 (2.9)	32.5 (1.9)	33.4 (0.7)
<i>non-autoclaved</i>									
Untreated	0.5 (0.1)	1.2 (0.2)	1.7 (0.4)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	16.1 (0.1)	14.5 (0.2)	13.4 (0.4)
$\text{NaNO}_2$	4.0 (0.3)	8.2 (2.5)	8.0 (1.0)	4.6 (2.0)	4.6 (0.2)	3.5 (0.3)	69.2 (2.7)	65.7 (1.5)	68.4 (4.5)
$\text{NaNO}_2$ + G	2.3 (0.2)	1.9 (0.4)	2.0 (0.4)	3.4 (2.0)	3.5 (0.0)	4.8 (1.4)	57.4 (1.6)	56.7 (2.8)	56.7 (2.5)

\* 300  $\mu\text{g C g}^{-1}$  soil

In the autoclaved soil a very high concentration of  $\text{NH}_4^+$  was found. Autoclaving has been reported to induce a large release of  $\text{NH}_4^+$  in the soil, mainly derived from the killed biomass which is further mineralized by the colonising microorganisms, but also deriving from the decomposition of the biologically resistant component of the organic matter which is solubilized by autoclaving (Salonius *et al.*, 1967; Powlson and Jenkinson, 1976a). A significant amount of  $\text{NO}_3^-$  was found in the autoclaved soil, after 24 h, presumably mainly deriving from the oxidation of the added  $\text{NaNO}_2$ , as in the autoclaved control, where plenty of extractable  $\text{NH}_4^+$  was available, the level of  $\text{NO}_3^-$  remained low (Table 4.4). This could indicate that the population of nitrite oxidisers had not only a high efficiency for utilising the substrate but also a potential for regrowth which was faster than that of the ammonium oxidizers.

In conclusion, the results clearly showed that no significant quantity of  $\text{N}_2\text{O}$  was produced by non-biological reactions of  $\text{NO}_2^-$  in the soil, consequently the high fluxes measured in the first experiment, when  $\text{NaNO}_2$  or  $\text{NaNO}_2$  and glucose were added to the soils, can be considered to have been of microbiological origin.

Results showed that different forms of N had a very different impact on  $\text{N}_2\text{O}$  emission in the two soils, suggesting the role of different microbial communities in the  $\text{N}_2\text{O}$  production in the two environments. The arable soil had a very low potential for  $\text{N}_2\text{O}$  emissions deriving from nitrifiable N as compared with the  $\text{N}_2\text{O}$  which was produced when the soil was provided with oxidized form of N and a carbon source (Table 4.5).

**Table 4.5** -  $\text{N}_2\text{O}$ -N emitted in 24 hours expressed as a percentage of the initially added N ( $70 \mu\text{g g}^{-1}$  soil) in various forms. Calculation are done on the average values.

Soil	<b><math>\text{N}_2\text{O}</math> emissions (%)</b>				
	$(\text{NH}_4)_2\text{SO}_4$	$\text{NaNO}_2$	$\text{NaNO}_2 + \text{glucose}$	$\text{Ca}(\text{NO}_3)_2 + \text{glucose}$	Peptone
<i>Woodland</i>	0.25	0.99	1.54	0.06	0.28
<i>Arable field</i>	0.35	0.45	2.40	2.17	0.18

These latter emissions were very high even though the soil could be considered to be predominantly aerobic, and probably derived from aerobic denitrification. The woodland soil showed a very low denitrification activity and a much higher  $\text{N}_2\text{O}$  production via  $\text{NH}_4^+$  oxidation and reduction of  $\text{NO}_2^-$  by some process mediated by autotrophic or heterotrophic nitrifiers as discussed previously.

## **4.2 EFFECT OF CYCLOHEXIMIDE ON PEPTONE INDUCED $\text{N}_2\text{O}$ EMISSIONS AND MINERAL N TRANSFORMATIONS IN THE WOODLAND SOIL**

### **4.2.1 INTRODUCTION**

As described in § 1.4, the role of autotrophic nitrifying bacteria in  $\text{N}_2\text{O}$  emissions is well documented, whereas the involvement of fungal populations in  $\text{N}_2\text{O}$  emissions has rarely been investigated and relates mainly to particular conditions such as acid coniferous soil (Bollag and Tung, 1972; Bleakley and Tiedje, 1982; Stroo *et al.*, 1986).

The objective of the present experiment was to evaluate the contribution of fungal activity to the overall nitrification activity and  $\text{N}_2\text{O}$  production induced by peptone addition in soil. As in the previous experiment (§ 4.1) peptone was observed to induced significant  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  production in the woodland soil but not in the arable soil, this second and more detailed experiment was limited only to the woodland soil

In the present experiment cycloheximide was used to selectively block any peptone-induced  $\text{N}_2\text{O}$  or  $\text{NO}_3^-$  production by eukaryotic soil microorganisms, which mainly consist of fungi in forest soils (see § 2.6.4 for details and comments on the selective inhibition technique by antibiotics). Cycloheximide was used in a range of concentrations from 0.5 to 9.5 mg of cycloheximide  $\text{g}^{-1}$  soil (dry weight), in order to

identify the most appropriate concentration of antibiotic for the studied soil. The limiting concentration of cycloheximide which can be found in the literature varies greatly, from 1 mg g<sup>-1</sup> (Anderson and Domsch, 1973) up to 80 mg g<sup>-1</sup> dry litter used by Kjølner and Struwe (1994).

It was initially assumed that the chosen concentrations of cycloheximide were not going to have a non-target effect on the bacterial component of the soil microflora and that the eukaryotic peptone-responsive component of the fungal population was also sensitive to the fungicide.

#### 4.2.2 *EXPERIMENTAL DESIGN*

Soil, sampled from the top 10 cm of woodland, was sieved (2 mm) and stored at room temperature (15-20°C). Water content was measured and the next evening cycloheximide (Sigma Chemical Co. Dorset, England) was added to the soil in quantities of 0, 0.5, 3.5, 5.5, 7.5 and 9.5 mg of antibiotic g<sup>-1</sup> dry soil. Talcum was used as a carrier medium (0.5 talcum 100g<sup>-1</sup> soil; Anderson and Domsch, 1973) for the antibiotic; samples were mixed thoroughly in plastic bags and left overnight.

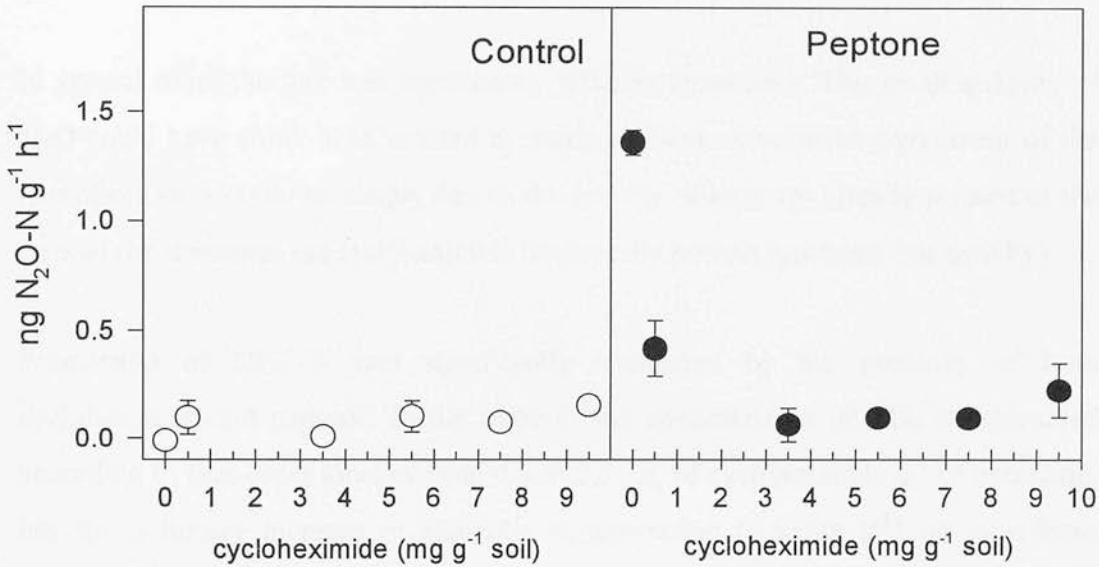
The next morning (after about 12 h) 70 µg of peptone-N g<sup>-1</sup> dry soil were added to the samples (except in the control). This lag in the time of application of cycloheximide and substrate was due to the fact that in previous experiments it was found that N<sub>2</sub>O production, after peptone addition, was almost immediate (about 1h), while the inhibiting effect of cycloheximide required several hours to produce significant effects. Consequently, a simultaneous application of antibiotic and peptone could lead to misleading results.

The soil was adjusted to 60% WFPS. Soil samples of 25 g each (3 replicates per each treatment) were incubated in 1 l air-tight jars and left at 25 °C for 24 hours.

At the end of the incubation, gas samples were withdrawn with 2 ml syringes and analyses by ECD gas chromatography. Subsamples of soil from each jar, were extracted for the analysis of mineral N. For details on methods see chapter 2.



In the absence of cycloheximide, the addition of peptone induced a significant increase in the  $\text{N}_2\text{O}$  production as compared with the control (Figure 4.2).



**Figure 4.2** -  $\text{N}_2\text{O}$  emission rates from the woodland soil treated with cycloheximide or cycloheximide plus peptone.

The peptone-induced  $\text{N}_2\text{O}$  flux was smaller than the one measured in the previous experiment (§ 4.1). However, they were both of the same order of magnitude. It is likely that slight differences in the microbial population size or activity could have occurred in between the two sampling events in the field, also influencing the microbial activity measured in the laboratory. Indeed, in short term incubations not enough time is available for the microbial population to change significantly so that the measured microbial activities in part reflect the situation of the microbial population before the incubation.

Addition of 0.5 mg of cycloheximide  $\text{g}^{-1}$  induced a significant drop in the peptone-induced  $\text{N}_2\text{O}$  production, which decreased from 1.36 to 0.41  $\text{ng N}_2\text{O-N g}^{-1} \text{ h}^{-1}$ . From 3.5 to 9.5 mg cycloheximide  $\text{g}^{-1}$ , the  $\text{N}_2\text{O}$  emissions from the peptone-treated samples did not differ significantly from the control. Neither in the control nor in the peptone treated samples did the presence of cycloheximide, which by itself could be a substrate for microbial activity and a source of N, induce any flux of  $\text{N}_2\text{O}$  (Figure 4.2).

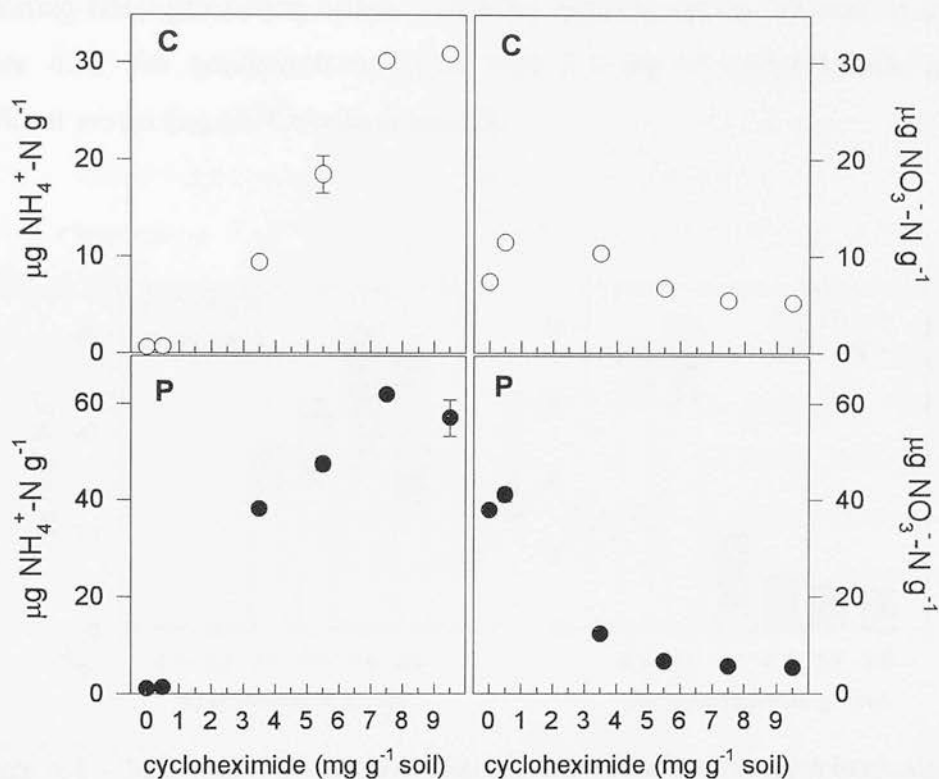
In several cases the flux was significantly different from zero. This small quantity of  $\text{N}_2\text{O}$  could have either been emitted by some antibiotic-insensitive component of the microflora or it could be simply due to the activity of enzymes already present at the time of the treatment (as cycloheximide blocks only protein synthesis, not activity).

Production of  $\text{NH}_4^+\text{-N}$  was significantly stimulated by the presence of both cycloheximide and peptone. In the control the concentration of  $\text{NH}_4^+\text{-N}$  increased according to first order kinetics from 0.5 to 7.5 mg of cycloheximide  $\text{g}^{-1}$  of antibiotic, but for a further increase in antibiotic concentration (9.5 mg  $\text{g}^{-1}$ ) no significant increase of  $\text{NH}_4^+\text{-N}$  was detected (Figure 4.3). At 7.5 and 9.5 mg of cycloheximide  $\text{g}^{-1}$ ,  $\text{NH}_4^+\text{-N}$  concentration remained stable around 30  $\mu\text{g N g}^{-1}$ .

When the sole peptone was added (zero cycloheximide) the concentration of  $\text{NH}_4^+\text{-N}$  in soil after 24 h was very low. Conversely, addition of cycloheximide (except 0.5 mg  $\text{g}^{-1}$ ) together with peptone induced a dramatic increase in  $\text{NH}_4^+\text{-N}$ , which reached 61.9  $\mu\text{g N g}^{-1}$  soil at 7.5 mg of cycloheximide  $\text{g}^{-1}$ . As for the control, 9.5 mg  $\text{g}^{-1}$  of antibiotic did not further increase  $\text{NH}_4^+\text{-N}$  concentration.

$\text{NH}_4^+\text{-N}$  extracted from the samples treated with peptone and antibiotic between 3.5 and 7.5 mg  $\text{g}^{-1}$  was more than twice as high as the  $\text{NH}_4^+\text{-N}$  extracted in the samples treated only with antibiotic. It was concluded that the observed difference was due to  $\text{NH}_4^+$  being mineralized or deaminated from peptone which, in the presence of cycloheximide was not immobilized and/or nitrified. 0.5 mg  $\text{g}^{-1}$  and 9.5 mg  $\text{g}^{-1}$  of

cycloheximide were probably too low and too high, respectively, to observe a proportional increase.



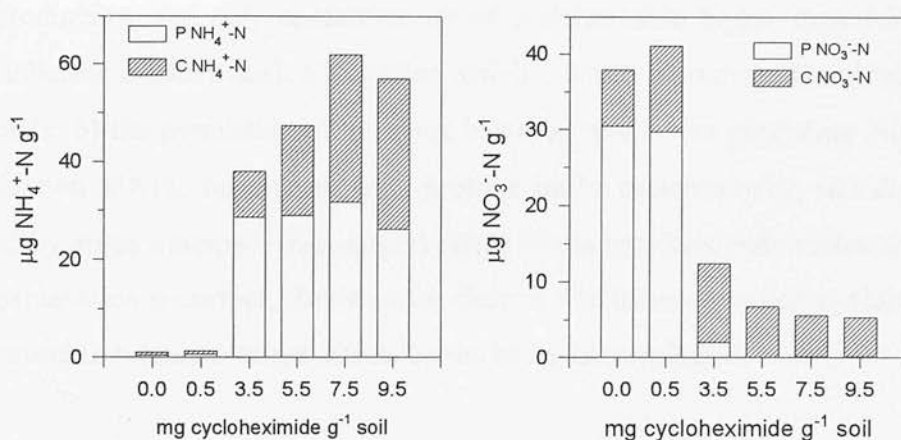
**Figure 4.3** - Mineral N extracted after 24 h incubation from the woodland soil treated with cycloheximide (C) or cycloheximide and peptone (P).

It is likely that cycloheximide blocked the processes which consume  $\text{NH}_4^+$  but not the processes which produce it, so that the  $\text{NH}_4^+$  derived from peptone, calculated to be around  $30 \mu\text{g N g}^{-1}$  (43% of the added peptone-N), could be detected, whereas it was not possible to identify it in the samples treated with peptone only.

From the observation of the  $\text{NO}_3^--\text{N}$  concentration, it is clear that the main process which was affected by the presence of cycloheximide was nitrification (Figure 4.3). Whereas the addition of peptone, with 0 or 0.5 mg of cycloheximide  $\text{g}^{-1}$ , induced a significant production of  $\text{NO}_3^-$ , for concentrations of antibiotic from 3.5 to 9.5  $\text{mg g}^{-1}$ ,

a drastic reduction of  $\text{NO}_3^-$  production was observed overall and the reduction of the  $\text{NO}_3^-$  production deriving from peptone N was even more drastic (Figure 4.3 and 4.4).

Zero or 0.5 mg of cycloheximide  $\text{g}^{-1}$  stimulated nitrification in the control, probably stimulating  $\text{NH}_4^+$  production without inhibiting significantly the nitrification process (Figure 4.3). For concentrations higher than 5.5 mg of cycloheximide  $\text{g}^{-1}$ , no significant production of  $\text{NO}_3^-$  was detectable.



**Figure 4.4** -  $\text{NH}_4^+$  and  $\text{NO}_3^-$  derived from peptone (P) and from cycloheximide plus background processes in the control (C).

The results obtained evidenced many points which did not have a clear answer.

First, cycloheximide had a drastic effect on the peptone induced  $\text{N}_2\text{O}$  emissions, which were already reduced almost to one third when 0.5 mg of cycloheximide  $\text{g}^{-1}$  was added to the soil. A concentration of 3.5 mg cycloheximide  $\text{g}^{-1}$  completely blocked the peptone-induced  $\text{N}_2\text{O}$  production.

This suggests that all the measured  $\text{N}_2\text{O}$  flux was derived from fungi and that whatever enzyme was needed for  $\text{N}_2\text{O}$  production was induced and not constitutive, otherwise a protein synthesis inhibitor such as cycloheximide could have not been effective.

An argument against this is that 3.5 mg of cycloheximide  $\text{g}^{-1}$  could have been already high enough to have non-target effects on other components of soil microflora, e.g. nitrifying bacteria. In this case the origin of the  $\text{N}_2\text{O}$  would be uncertain.

Second, cycloheximide reduced drastically the peptone-induced  $\text{NO}_3^-$  production at 3.5  $\text{mg g}^{-1}$  and  $\text{NO}_3^-$  production in the control at 5.5  $\text{mg g}^{-1}$  (Figure 4.4). At 7.5  $\text{mg g}^{-1}$ , the  $\text{NO}_3^-$  present in the soil was even lower than the  $\text{NO}_3^-$  level at time zero (6.3  $\mu\text{g NO}_3^- \text{-N g}^{-1}$ ) in both the control and the peptone treated samples.

Possible explanations could be: a) fungi were the only organisms responsible for the  $\text{NO}_3^-$  production, and only concentrations of cycloheximide higher than 5.5  $\text{mg g}^{-1}$  were sufficient to block such nitrification activity, which moreover should have been induceable; b) the population of nitrifying bacteria, which was producing  $\text{NO}_3^-$  from the oxidation of  $\text{NH}_4^+$  mineralized from peptone and/or cycloheximide, was drastically reduced by some unknown (non-target) effect of the cycloheximide molecule. If the latter explanation is correct, then it is not clear at which concentration cycloheximide was starting to have non-target effects on the bacterial population.

However, the same concentrations of cycloheximide which blocked nitrification, did not stop mineralization. Indeed, cycloheximide stimulated  $\text{NH}_4^+$  production in soil, probably because the antibiotic itself acted as a substrate for the microorganisms. It has been reported that cycloheximide (as well as streptomycin) can be mineralized by soil microorganisms (Stamatiadis *et al.*, 1990; Landi *et al.*, 1993; Badaluco *et al.*, 1994).  $\text{NH}_4^+$  concentration in soil did not further increase between 7.5 and 9.5  $\text{mg g}^{-1}$  of cycloheximide  $\text{g}^{-1}$ .

This suggests either that heterotrophic microorganisms were less sensitive to cycloheximide than nitrifiers, or that they were equally sensitive and that the production of  $\text{NH}_4^+$  was mainly due to pre-existent extra-cellular enzymes.

Regarding this point it is worth noting that for increasing concentrations of cycloheximide, the  $\text{NH}_4^+$  deriving from peptone remained stable at about 30  $\mu\text{g N g}^{-1}$  (Figure 4.4), whereas the  $\text{NH}_4^+$  deriving from the cycloheximide increased up to 7.5  $\text{mg cycloheximide g}^{-1}$ . This suggests the presence of two different microbial

populations or enzymatic pathways, one of which was able to mineralize peptone and was insensitive to increasing concentrations of cycloheximide (in this case extra-cellular enzyme activity could be a possible hypothesis) and a second population, which was able to mineralize the complex molecule of cycloheximide and which could have been sensitive only to high concentrations of antibiotic.

Stamatiadis *et al.* (1990) observed an increase in total and active bacterial population in a soil amended with 2 mg g<sup>-1</sup> cycloheximide, suggesting that heterotrophic bacteria were not disturbed by the presence of fungicide (at that concentration) and were able to utilise it as a substrate. We do not know, however, if this would apply at higher concentrations of cycloheximide.

It is clear that several points need further investigation:

- Is the peptone-induced N<sub>2</sub>O really derived from fungal activity ? To answer this question we need to understand if the concentrations of cycloheximide which blocked N<sub>2</sub>O production could have had non-target effects on other components of the soil microflora.
- What could be the process of N<sub>2</sub>O formation by fungi ? It was observed in the previous experiment that peptone-induced N<sub>2</sub>O emissions in the woodland were blocked by 0.1% C<sub>2</sub>H<sub>2</sub>, suggesting an autotrophic rather than an heterotrophic route.
- What is the limiting concentration of cycloheximide which affects bacterial nitrification ?
- What is the influence of cycloheximide on mineralization, and what are the roles of fungi and bacteria in the mineralization of both antibiotic and the peptone ?



## FURTHER STUDIES ON THE CONTRIBUTION OF FUNGI TO N<sub>2</sub>O RELEASE, MINERALIZATION AND NITRIFICATION IN SOIL

### 5.1 EFFECTIVENESS OF CYCLOHEXIMIDE IN DIFFERENTIATING BETWEEN FUNGAL AND BACTERIAL N<sub>2</sub>O AND NO<sub>3</sub><sup>-</sup> PRODUCTION<sup>1</sup>

#### 5.1.1 INTRODUCTION

The following experiment was intended to further investigate the contribution of the fungal component of soil microflora to N<sub>2</sub>O emissions and nitrification in the woodland soil and the effectiveness of the antibiotic block technique for this purpose. A previous experiment (§ 4.1) had shown that peptone, added to the woodland soil, induced a significant production of N<sub>2</sub>O and NO<sub>3</sub><sup>-</sup> but that 0.1% C<sub>2</sub>H<sub>2</sub> blocked such production. This could be evidence for autotrophic nitrification as a source for both the nitrogenous oxides. However, it was found that the fungicide cycloheximide blocked N<sub>2</sub>O and NO<sub>3</sub><sup>-</sup> production as well. Consequently, this raised the question of whether the use of cycloheximide was appropriate to differentiate between fungal and bacterial N<sub>2</sub>O and NO<sub>3</sub><sup>-</sup> production and which could be the pathway for such production. As shown in chapter 1, the involvement of heterotrophic microorganisms<sup>1</sup> in N<sub>2</sub>O emissions has been rarely investigated, and mainly refers to particular

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<sup>1</sup> A paper entitled "Effect of cycloheximide on N<sub>2</sub>O and NO<sub>3</sub><sup>-</sup> production in a forest and an agricultural soil" based on the results presented in this paragraph has been accepted in November 1997 on *Biology and Fertility of Soils*.

conditions such as acid coniferous soil. Moreover, few pathways of fungal nitrification have been proposed but no conclusive evidence has been provided. Critical comments on the available information on the antibiotic block technique and on the use of  $C_2H_2$  have been given in § 2.6 and § 2.5, respectively.

The effect of cycloheximide (at concentrations used to block peptone-induced nitrification) on soil microbial biomass, soil  $\alpha$ -amino-N, net mineralization and net nitrification was followed for 2 days, starting from the time of application, to test the possibility of a biocidal action of cycloheximide on the overall microbial population, which could have resulted in non-target effects on the bacterial component of the heterotrophic and autotrophic nitrifiers. In the case of a drastic and non-target poisoning effect of cycloheximide on the microbial community, it could be expected to observe an almost immediate decrease of microbial biomass and activity and an increase in  $\alpha$ -amino-N, which has a very low concentration in the soil but quite a high concentration in the microbial cytoplasm. Few studies have been conducted on the activity and degradation of cycloheximide in soil (Landi *et al*, 1993; Badalucco *et al*, 1994) and generally the changes have been observed only after a minimum period of 1 day.

### 5.1.2 EXPERIMENTAL DESIGN

Soil was sampled from the top 10 cm of the woodland soil, sieved (2 mm) and stored at room temperature (20 °C) overnight.

*Experiment 1.* Soil was preincubated (12 h) with 0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 mg of cycloheximide  $g^{-1}$  soil dry weight as described in § 4.2.2. The next morning, 70  $\mu g$  of peptone-N  $g^{-1}$  dry soil were added to the soil. The soil was adjusted to 60% of WFPS and samples of 50 g each were incubated in 1 l air-tight jars (§ 2.3). Immediately after the lids were closed, 0.1 % (v/v)  $C_2H_2$  was added by syringe to the jars in order to block the activity of ammonia-monooxygenase. For the control and for each cycloheximide concentration applied, three replicates with  $C_2H_2$  and three

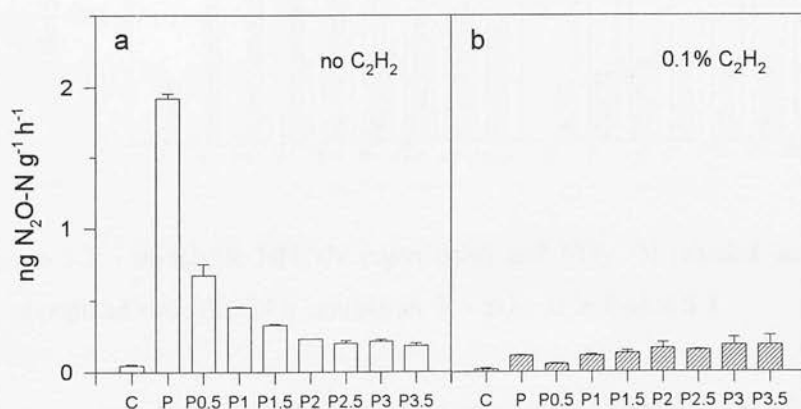
without  $C_2H_2$  were incubated at 25 °C. After about 24 h, gas samples for the measurement of  $N_2O$  were taken with 2 ml greased glass syringes and immediately analysed by ECD gas chromatography. At the same time soil was extracted for the analysis of mineral N.

*Experiment 2.* Woodland soil was mixed with 0, 0.5, 1.5, 2.5 and 5.0 mg of cycloheximide  $g^{-1}$  soil (dry weight) as described in § 4.2.2. The soil was adjusted to the 60% of WFPS. Soil samples of 20 g each (in triplicate) were incubated in 300 ml pots, which were firmly closed and stored at 25 °C. Soil was sampled after 0, 1, 3, 5, 9, 23 and 47 h of incubation and was extracted with 1 M KCl for the analysis of mineral N and  $\alpha$ -amino N. At 0, 24 and 48 h, subsamples of 10 g of soil, from each pot, were collected for the measurement of microbial biomass. For laboratory methods and statistical analysis see chapter 2.

### 5.1.3 RESULTS AND DISCUSSION

#### Effect of fungicide on $N_2O$ fluxes and nitrate production

Peptone stimulated  $N_2O$  and  $NO_3^-$  production (Figure. 5.1a, 5.2a).

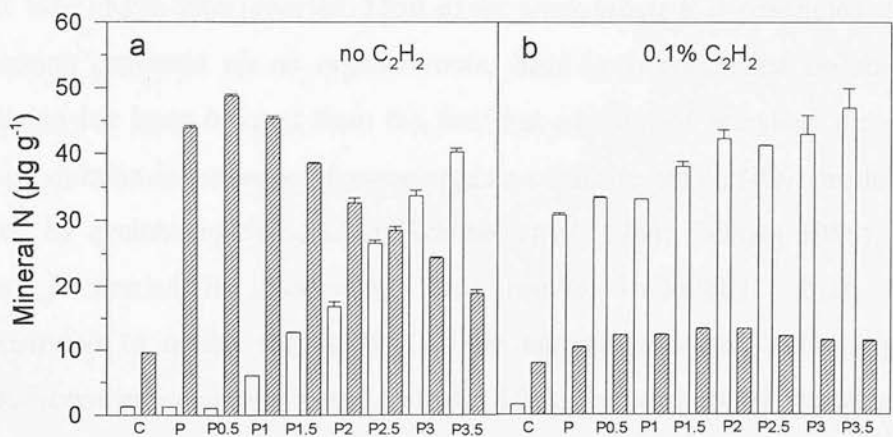


**Figure 5.1** -  $N_2O$  emission rates from the woodland soil untreated (C), treated with peptone (P) or with peptone plus increasing concentration of cycloheximide expressed as  $mg\ g^{-1}$  dry soil (numbers on X-axes), in presence of 0 (a) or 0.1% (b)  $C_2H_2$ .

A 10-fold increase in the N<sub>2</sub>O emission rate was observed where peptone was added, compared with the control (Figure 5.1a).

The addition of antibiotic to the soil had a clear effect on N<sub>2</sub>O emissions. In the presence of 0.5 mg g<sup>-1</sup> of cycloheximide the emission was reduced by two-thirds, and the inhibition was 90% at 2 mg g<sup>-1</sup> (Figure 5.1a). When 0.1% C<sub>2</sub>H<sub>2</sub> was added to the woodland soil treated only with peptone, the N<sub>2</sub>O emission rate was reduced by about 94%, dropping immediately to 0.11 ng N<sub>2</sub>O-N g<sup>-1</sup> h<sup>-1</sup> (Figure 5.1b). From 0.5 to 1.5 mg g<sup>-1</sup> of cycloheximide, the presence of C<sub>2</sub>H<sub>2</sub> significantly reduced the N<sub>2</sub>O emissions below those occurring with cycloheximide alone; from 2 to 3.5 mg g<sup>-1</sup> of cycloheximide the C<sub>2</sub>H<sub>2</sub> had no significant effect (Figure 5.1b).

Mineral N concentrations in the different treatments are shown in Figure 5.2.



**Figure 5.2** - Available NH<sub>4</sub><sup>+</sup>-N (open bars) and NO<sub>3</sub><sup>-</sup>-N (shaded bars) measured in the woodland soil after 24 h incubation. X - axis: as in Figure 5.1.

The presence of peptone stimulated the production of NO<sub>3</sub><sup>-</sup>. After 24 h incubation, no accumulation of NH<sub>4</sub><sup>+</sup> was observed where only peptone was added. The addition of increasing concentrations of cycloheximide resulted in an increasing accumulation of NH<sub>4</sub><sup>+</sup> and a decrease in NO<sub>3</sub><sup>-</sup> production.

When  $C_2H_2$  was added together with peptone, almost all the production of  $NO_3^-$  was blocked at the control level, while the concentration of  $NH_4^+$  increased (Figure 5.2b). This  $NH_4^+$  accounted for about 85% of the  $NO_3^-$  produced from peptone in the absence of  $C_2H_2$ ; only half of the remaining 15% , though, was found as  $NO_3^-$ .

Comparing the presented results with results in the literature, they appear somewhat contradictory. On the one hand, fungi (considered heterotrophic nitrifiers) may seem mainly responsible for  $N_2O$  emissions in the woodland soil, as indicated by cycloheximide inhibition of  $N_2O$  flux even at low concentrations. On the other hand, the inhibition of  $N_2O$  production by  $C_2H_2$  suggests that ammonia monooxygenase, typical of autotrophic nitrifiers, is in some way involved in the process.

It is generally believed that fungi are heterotrophic nitrifiers which produce  $NO_3^-$  via an organic pathway. However, experiments done on pure cultures of fungi are very scarce and both inorganic (Aleem *et al.* 1964) and organic pathways (Doxtader 1965; Wood 1987) have been reported. Most of the work where it is concluded that fungal nitrification proceeds via an organic route, have been conducted on soil and the conclusion has been inferred from the fact that addition of acetylene did not block  $NO_3^-$  production in presence of some organic substrate, while  $NO_3^-$  production was blocked by cycloheximide addition (Schimel *et al.* 1984; Killham 1986). However caution is needed in interpreting these results. Undoubtly  $C_2H_2$  has been demonstrated to inhibit the activity of the enzyme ammonia monooxygenase in *Nitrosomonas europaea* (Hynes and Knowles 1982; Knowles 1990), which is the most common autotrophic nitrifying bacterium, but less evidence has been provided that heterotrophic nitrifiers, including fungi, are not sensitive to  $C_2H_2$ . Of all the literature which is more commonly cited,  $C_2H_2$  has only been tested in two works on heterotrophic microorganisms, once on *Arthrobacter* sp. (Hynes and Knowles 1982) and once on a strain of *Aspergillus flavus* (Schimel *et al.* 1984) (see also § 2.5).

Fungi have been reported to be able to nitrify (Schmidt 1960; Van Gool and Schmidt 1973) in the presence of peptone, with production of  $NO_3^-$ , and it was suggested that

nitrification activity functioned as an endogenous respiration to support some growth or cell functions, with very small energy production, when no other substrate is readily available or when the C/N ratio of the substrate is very low. Aleem *et al.* (1964) reported fungal nitrification of  $\text{NH}_4^+$  to  $\text{NO}_3^-$  proceeding through an inorganic pathway. The possibility that the fungal population could synthesise a similar enzyme to ammonia monooxygenase to nitrify the  $\text{NH}_4^+$  accumulated via an inorganic pathway, similar to the bacterial one, could explain the inhibition of  $\text{N}_2\text{O}$  emissions by cycloheximide (block of the enzyme synthesis) and 0.1%  $\text{C}_2\text{H}_2$  (block of the enzyme function). Though the presence of ammonia monooxygenase has not been demonstrated in fungi, some evidence of a nitrification pathway which resembles that of autotrophs in many ways has been produced for some heterotrophic bacteria (Kurokawa *et al.* 1985; Robertson *et al.* 1988; Robertson and Kuenen 1990).

In a similar experiment carried out on the arable soil (Appendix III), the complete lack of  $\text{N}_2\text{O}$  production, and the very small rate of  $\text{NO}_3^-$  production after peptone addition, could support the idea of a major role for the fungal population in peptone degradation and  $\text{N}_2\text{O}$  or  $\text{NO}_3^-$  production. Due to its characteristics (very low C and water content, no input of manure or fresh litter, and almost no structure), the arable soil probably support a much lower fungal biomass as compared with the woodland soil.

A second explanation for the presented results could be that autotrophic bacteria were the responsible for  $\text{N}_2\text{O}$  production and that cycloheximide had a non target effect on bacteria starting from  $0.5 \text{ mg g}^{-1}$  dry soil.

#### Influence of cycloheximide on soil microbial biomass, mineralization and nitrification

Values of biomass ninhydrin-N in the control were almost stable at around  $38 \text{ } \mu\text{g g}^{-1}$ , throughout the incubation period (Table 5.1). These values are in the same range as those found by Amato and Ladd (1988) for fumigated forest and wheat soils, and are



2 to 10 times higher than values found for a whole series of fumigated soils by Sparling and Zhu (1993).

**Table 5.1** - Biomass ninhydrin-N ( $\mu\text{g g}^{-1}$  dry soil) from the woodland soil treated with different concentrations of cycloheximide up to  $5 \text{ mg g}^{-1}$  soil, measured at 0, 24 and 48 hours of incubation.

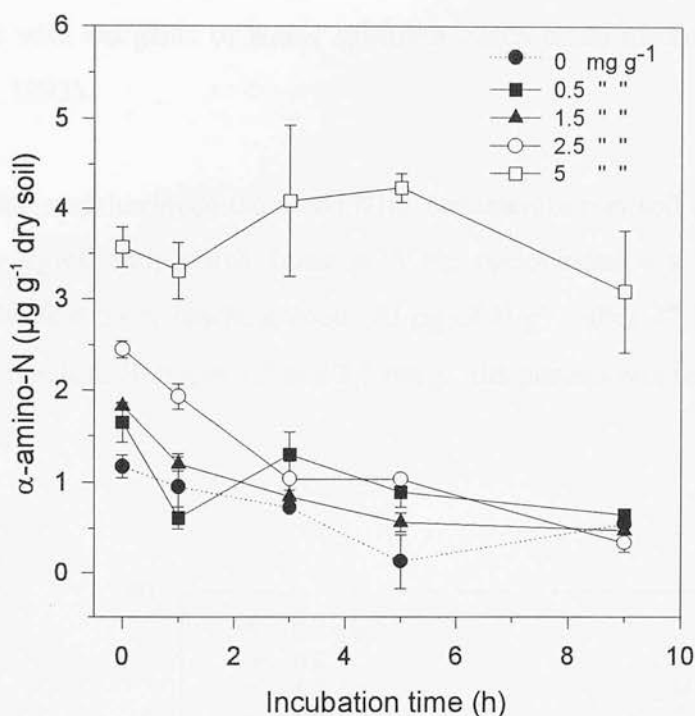
mg cycloheximide $\text{g}^{-1}$	Biomass ninhydrin-N ( $\mu\text{g g}^{-1}$ dry soil)				
	0	0.5	1.5	2.5	5
Incubation time (h)					
0	37.4(ab,1)	40.8(ab,2)	42.8(b,2)	38.7(ab,2)	34.3(a,2)
24	39.7(c,1)	37.8(c,12)	37.3(c,12)	34.5(b,12)	27.2(a,1)
48	37.2(c,1)	36.2(c,1)	33.9(bc,1)	30.8(b,1)	25.2(a,1)

Note: In subscripts different letters and numbers indicate significant ( $P < 0.05$ ) differences among values in columns and rows, respectively.

At 0.5 and  $1.5 \text{ mg cycloheximide g}^{-1}$  a significant, but not drastic, decrease of microbial biomass was observed only after 48 h. At  $2.5 \text{ mg cycloheximide g}^{-1}$ , microbial biomass significantly decreased already at 24 h, while  $5 \text{ mg cycloheximide g}^{-1}$  induced a drastic reduction of microbial biomass at 24h and 48h.

The slight decrease of microbial biomass from 0 to  $2.5 \text{ mg cycloheximide g}^{-1}$  could be explained as an inhibition of fungal biomass turnover, by the block of fungal protein synthesis, which could produce an observable biomass decrease only after 48 h. Whereas, concentrations of cycloheximide  $> 2.5 \text{ mg g}^{-1}$  could be toxic for soil microorganisms. In this case the toxicity may not be so specific as the protein synthesis block, and consequently a non-target effects on soil microorganisms other than fungi could lead to a more drastic decrease of microbial biomass already in the first 24h. A disruptive effect on microbial biomass could results in cellular lysis with consequent release of low molecular weight N compounds of the cytoplasm. Indeed, a significant increase of  $\alpha$ -amino N was measured in soil at time zero, only at  $2.5$  and  $5 \text{ mg cycloheximide g}^{-1}$  (Fig. 5.3). At  $2.5 \text{ mg cycloheximide g}^{-1}$ ,  $\alpha$ -amino-N in soil

declined to the control level after 9 h, but at 5 mg g<sup>-1</sup> it remained consistently high throughout the 9 h incubation. After 24 h the values of  $\alpha$ -amino-N in all the samples, where cycloheximide was added, did not differ significantly from the control (data not shown).



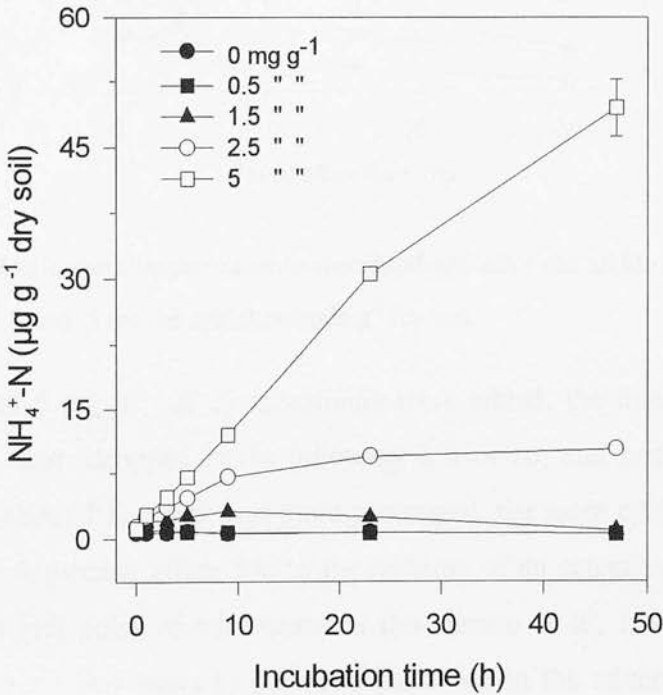
**Figure 5.3** -  $\alpha$ -amino N content in woodland soil during the first 9 hours of incubation, after the addition of 0, 0.5, 1.5, 2.5 and 5 mg of cycloheximide g<sup>-1</sup> dry soil.

Badalucco *et al.* (1994) measured a similar increase of extractable C and N in a forest soil after 48 h incubation with 2 mg g<sup>-1</sup> of cycloheximide, accompanied by a parallel decrease of microbial biomass, and concluded that lysis of killed cells could have occurred. At 2 mg g<sup>-1</sup> of cycloheximide, Landi *et al.* (1993) observed a reduction in biomass-N in a forest and a wheat soil comparable to the reduction found in our woodland soil at 2.5 mg g<sup>-1</sup> of cycloheximide. Biocidal effects could be much less specific than the block of protein synthesis and could be the explanation for observed

effects of the antibiotic on non-target groups (Ingham and Coleman 1984; Landi *et al.* 1993).

The sensitivity of the microorganisms to poisoning concentrations of cycloheximide could depend on several factors, such as the composition of the soil microbial community, the presence of other stress factors, and the possibility of interactions of the antibiotic with inorganic or humic substrate which could inactivate the molecule (Landi *et al.*, 1993).

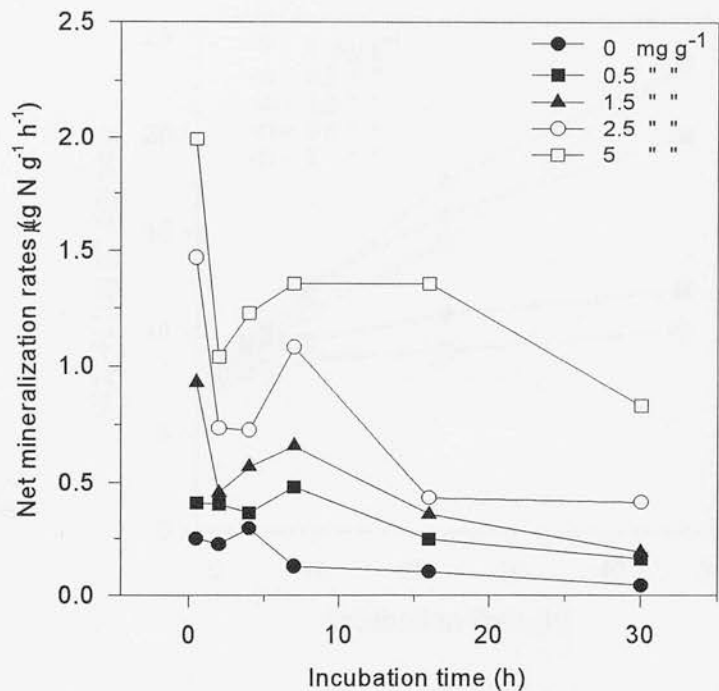
Addition of the cycloheximide increased  $\text{NH}_4^+$  concentration in soil (Figure 5.4). This increase was significantly much faster at 5 mg cycloheximide  $\text{g}^{-1}$ , and proceeded linearly for the first 24 h, reaching about 30  $\mu\text{g}$  of  $\text{N g}^{-1}$ ; after 47 h the plateau had still not been reached. Between 1.5 and 2.5 mg  $\text{g}^{-1}$  the plateau was reached after about 9 h.



**Figure 5.4** - Cumulative  $\text{NH}_4^+$ -N in woodland soil at different times after the addition of 0, 0.5, 1.5, 2.5 and 5 mg of cycloheximide  $\text{g}^{-1}$  dry soil.

The net N mineralization rates, calculated for each time interval as  $(\Delta\text{NH}_4^+ + \Delta\text{NO}_3^-)/\Delta t$ , were not significantly correlated with the  $\text{NH}_4^+\text{-N}$  measured in soil at the same time intervals, except for the control ( $r = 0.833$ ,  $P < 0.02$ ).

Where 0 or 0.5  $\text{mg g}^{-1}$  of cycloheximide were added, the maximum mineralization rate was reached after about 3 and 7 h, respectively (Figure 5.5).

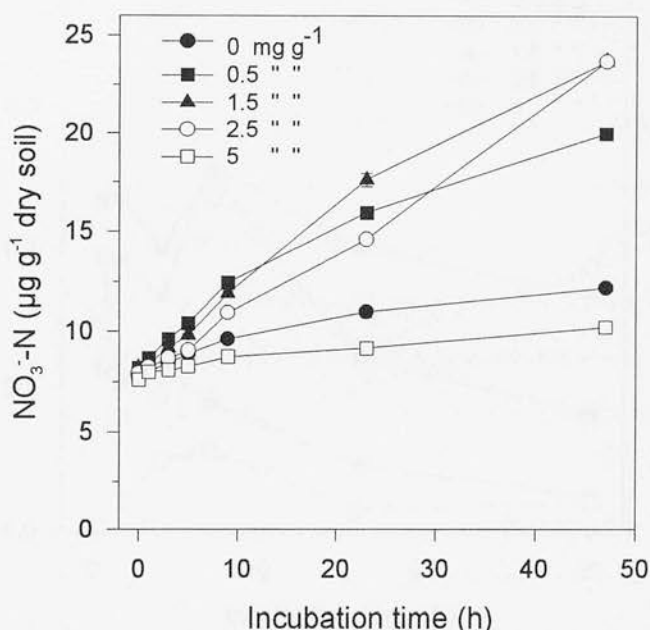


**Figure 5.5** - Net mineralization rates in woodland soil after the addition of 0, 0.5, 1.5, 2.5 and 5  $\text{mg g}^{-1}$  of cycloheximide  $\text{g}^{-1}$  dry soil.

When 1.5, 2.5 and 5  $\text{mg g}^{-1}$  of cycloheximide were added, the mineralization was faster in the first hour, dropped in the following 2 h or so, and had a second, but lower, peak after about 7 h, which was more prolonged, the more cycloheximide was added to the soil. A priming effect due to the addition of an organic substrate could have stimulated a first pulse of mineralization (Badalucco *et al*, 1994; Landi *et al*, 1993) followed after a few hours by a second pulse due to the mineralization of the biocide-killed cells or of the cycloheximide molecule. The cycloheximide molecule

contains about 5% N, so the addition of 0, 0.5, 1.5, 2.5 and 5 mg g<sup>-1</sup> of cycloheximide corresponds to an addition of 0, 25, 75, 125 and 250 µg N g<sup>-1</sup>, respectively.

The addition of cycloheximide had a drastic effect on NO<sub>3</sub><sup>-</sup> production (Figure 5.6).

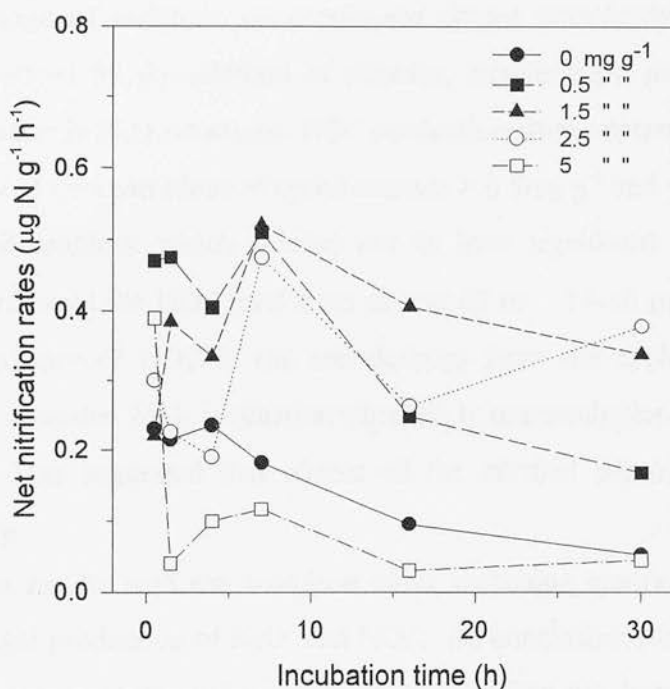


**Figure 5.6** - Cumulative NO<sub>3</sub><sup>-</sup>-N in woodland soil at different times after the addition of 0, 0.5, 1.5, 2.5 and 5 mg of cycloheximide g<sup>-1</sup> dry soil.

From 0.5 to 2.5 mg g<sup>-1</sup>, cycloheximide stimulated NO<sub>3</sub><sup>-</sup> production (Fig. 5.6). However, NO<sub>3</sub><sup>-</sup> production at 0.5 mg cycloheximide g<sup>-1</sup> was not significantly different from production at 1.5 and 2.5 mg cycloheximide g<sup>-1</sup>, probably because the cycloheximide was acting as an inhibitor of the synthesis of extra-enzymes needed to nitrify the NH<sub>4</sub><sup>+</sup>-N made available by mineralization. At 5 mg cycloheximide g<sup>-1</sup>, NO<sub>3</sub><sup>-</sup> production was below control level.

Net nitrification rates were calculated as  $\Delta\text{NO}_3^-/\Delta t$ , as no NO<sub>3</sub><sup>-</sup> was lost from the system as leachate and only a minor part as gaseous N. The rates, as for net

mineralization, reached a maximum at 5 h in the control and at 7 h in the other treatments, except for 5 mg g<sup>-1</sup> of cycloheximide, where the rate decreased after only 1 h (Figure 5.7), confirming that concentrations higher than 2.5 mg cycloheximide g<sup>-1</sup>, were having a detrimental effect on the overall nitrifier population.



**Figure 5.7** - Net nitrification rates in woodland soil after the addition of 0, 0.5, 1.5, 2.5 and 5 mg of cycloheximide g<sup>-1</sup> dry soil.

Nitrification data indicated that 5 mg of cycloheximide were inducing a negative effect on nitrifier population, which probably was more drastic than the simple inhibition of fungal protein synthesis. In the latter case, we would have observed a block in the NO<sub>3</sub><sup>-</sup> production, which would have been in the same range of the control treatment, as observed for 1.5 and 2.5 mg of cycloheximide g<sup>-1</sup>. On the contrary, net nitrification with 5 mg of cycloheximide resulted significantly much lower than in the control, thus suggesting that this concentration of antibiotic was having a disruptive effects on the nitrifiers population. This would be in accord with the data on biomass and α-amino-N.



In conclusion, the results seem to support the idea that in the woodland soil cycloheximide used at concentrations  $< 2 \text{ mg g}^{-1}$  would have an inhibiting action but not a biocidal effect, so that to a good approximation a selective effect of cycloheximide on soil eukaryotes can be expected.

This same range of antibiotic concentrations almost completely blocked the  $\text{N}_2\text{O}$  emissions, induced by the addition of peptone, suggesting a potential role of the fungal population in  $\text{N}_2\text{O}$  emissions.  $\text{NO}_3^-$  production from peptone-N was about  $30\text{--}35 \mu\text{g N g}^{-1}$ , and concentrations of cycloheximide  $> 0.5 \text{ mg g}^{-1}$  and  $< 2.5 \text{ mg g}^{-1}$  (which is a range of antibiotic which seemed not to have significant non-target effects) significantly reduced the  $\text{NO}_3^-$  level from almost 60 to  $\sim 25\text{--}30 \mu\text{g N g}^{-1}$ , which was about the quantity of  $\text{NO}_3^-$  in the soil deriving from the cycloheximide addition (Figure 5.6: consider 24 h incubation plus 12 h pre-incubation to calculate  $\text{NO}_3^-$  production). This suggested that almost all the nitrified peptone-N derived from fungal activity.

However, the results with the acetylene block technique contrast with the current views on fungal production of  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$ . As conclusive evidence on the effect of cycloheximide on autotrophic nitrifying bacteria has not been provided, a non-target effect of cycloheximide on nitrifying bacteria cannot be excluded.

## **5.2 EFFECTS OF CYCLOHEXIMIDE ON PEPTONE-N MINERALIZATION, $\text{N}_2\text{O}$ EMISSIONS AND NITRIFICATION IN THE FUMIGATED WOODLAND SOIL**

### **5.2.1 INTRODUCTION**

In chapter 4, § 4.5, it was shown that, whereas nitrification and  $\text{N}_2\text{O}$  emission were completely blocked by the addition of high concentrations of cycloheximide ( $< 5.5 \text{ mg g}^{-1}$ ), the mineralization rates were still increasing at  $7.5 \text{ mg}$  of cycloheximide  $\text{g}^{-1}$ .

Moreover, different trends were observed for the mineralization of peptone-N and cycloheximide-N. The role of the fungal and bacterial populations in these processes was unclear. Several hypotheses were put forward, which included the possibility of some role of extra-cellular pre-existent enzymes in the mineralization of the substrates.

The experiment in this section was designed to test the effect of cycloheximide on mineralization,  $N_2O$  emission and nitrification, comparing a fumigated with an unfumigated soil. Fumigation with chloroform has been proved to reduce the microbial population drastically, as the chloroform molecule interacts with the cell membrane, inducing cell lysis (Powlson and Jenkinson, 1976a). This would help, first, to compare the effect of cycloheximide on a pre-existent population (unfumigated soil) with the effect of cycloheximide on a growing population (after fumigation and chloroform removal) in active protein synthesis; second, to investigate the role of extra-cellular enzymes in peptone mineralization. For this latter purpose increasing concentrations of peptone were added to the soil. It was expected that in the fumigated soil, treated with peptone and cycloheximide, a complete block of mineralization would indicate that a growing fungal population was responsible for peptone mineralization; a partially inhibited or non-inhibited mineralization, which remained constant for increasing concentration of peptone addition, would indicate a pre-existent enzymatic activity as the main mechanism of peptone mineralization; a cycloheximide-insensitive activity of mineralization which, however, increased with increasing concentrations of peptone, would indicate a major role of bacteria in peptone mineralization.

The concentration used was  $7.5 \text{ mg of cycloheximide g}^{-1}$ ; at this concentration, the overall mineralization activity in the soil was still increasing while the nitrification activity was completely blocked (§ 4.5), making it possible to distinguish how much of the added N was mineralized. Any eventual immobilization was not measured and consequently not considered in the calculation, so that reference to mineralization mainly refers to net mineralization.

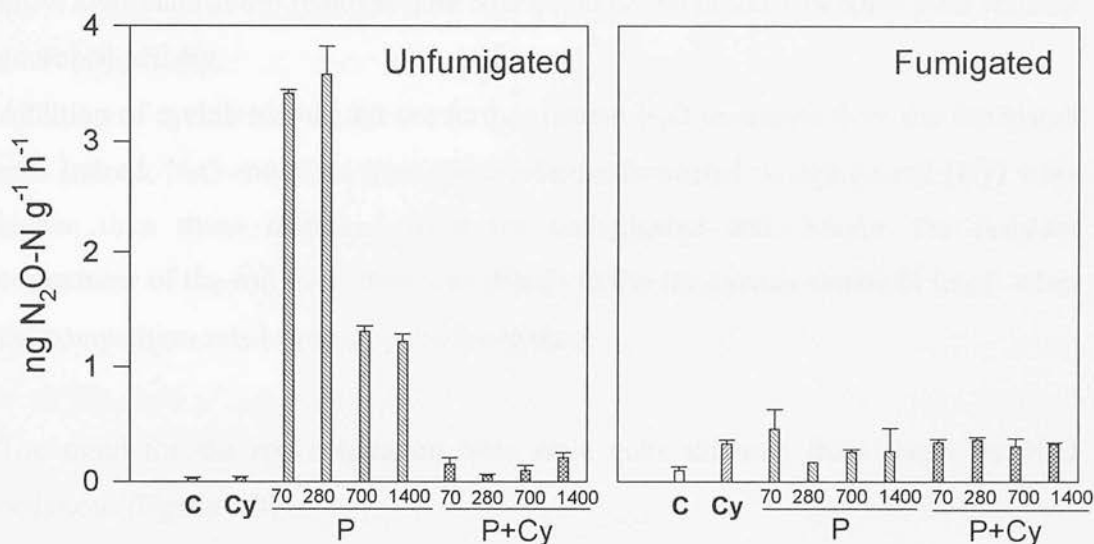
### 5.2.2 EXPERIMENTAL DESIGN

The woodland soil was sampled and sieved as described in § 5.1.2. Part of the soil was then fumigated, by putting it in a desiccator with 50 ml of chloroform, and evacuating the desiccator till the chloroform was boiling. The soil was left exposed to the chloroform atmosphere for 24 h. Chloroform was then removed by repeatedly evacuating the desiccator, after having removed the beaker with the remaining chloroform. The other part of the soil was left unfumigated in plastic bags in the same room where the desiccator was placed. Water content was then measured. Next evening, fumigated and unfumigated soils were pre-incubated (12 h) with 7.5 mg of cycloheximide (as previously described, § 4.2.2), and a control sample was left aside. The following day 70, 280, 700 and 1400  $\mu\text{g N g}^{-1}$  were added as peptone. Soil was brought to the 60% WFPS. Three replicates were used for each treatment: (C) control (fumigated or unfumigated), (Cy) soil plus cycloheximide (unfumigated or fumigated), (P) soil plus peptone at different concentrations (unfumigated or fumigated), (P+Cy) soil (unfumigated and fumigated) plus peptone (different concentrations) plus cycloheximide.

Soil (25 g) was incubated in 1 l air-tight jars for 24 h at 25°C. At the end of the incubation period, two sequential gas samples, of 2 ml each, were taken as described previously for the analysis of  $\text{N}_2\text{O}$  and  $\text{CO}_2$ , and soil was extracted for the analysis of mineral N.

### 5.2.3 RESULTS AND DISCUSSION

Addition of 70  $\mu\text{g}$  of peptone-N  $\text{g}^{-1}$  to the unfumigated soil induced a significant increase in  $\text{N}_2\text{O}$  with respect to the control (Figure 5.8). The same was observed for 280  $\mu\text{g}$  of peptone-N  $\text{g}^{-1}$ , though the addition did not further increase  $\text{N}_2\text{O}$  emission rates.



**Figure 5.8** - N<sub>2</sub>O emission rates from the woodland soil, either unfumigated or fumigated, untreated (C), treated with cycloheximide (Cy), or with peptone (P) at different concentrations (mg N g<sup>-1</sup>), or with peptone and cycloheximide (P+Cy).

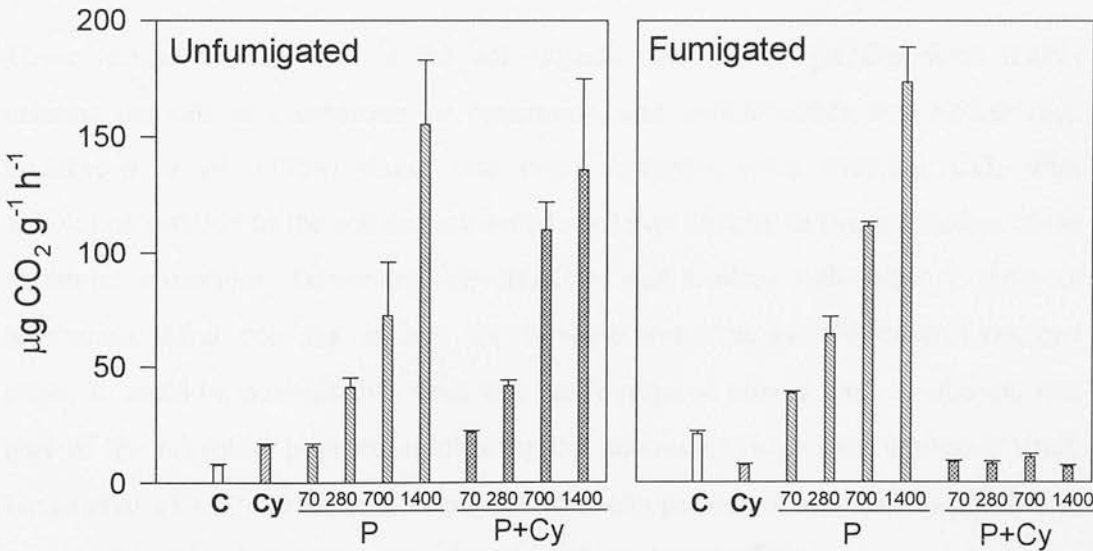
When 700 and 1400  $\mu\text{g}$  peptone-N g<sup>-1</sup> were added, the emission rates were only a third of those with 70 and 280  $\mu\text{g}$  N g<sup>-1</sup>. As can be seen in Figure 5.8, the standard error of the mean of the three replicates was very small and so the extremely low N<sub>2</sub>O emission rates measured when 700 and 1400  $\mu\text{g}$  peptone-N g<sup>-1</sup> were added cannot be attributed to some leaking jars or syringes. A possible explanation for the observed result is that the extremely high concentrations of NH<sub>4</sub><sup>+</sup> found in the soil (see below) could have partially inhibited the mechanisms involved in N<sub>2</sub>O production. Addition of cycloheximide together with peptone to the unfumigated soil almost completely blocked the N<sub>2</sub>O flux, as noted in previous experiments (§ 4.2, § 5.1).

When peptone was added to the fumigated soil, the emissions were quite low but still significantly different from zero. Fumigation usually kills almost all the

microorganisms present in the soil, but a few microorganisms situated in protected microsites, or spore-forming microorganisms, can resist the chloroform treatment and grow after chloroform removal. The  $N_2O$  could be the product of some such residual microbial activity.

Addition of cycloheximide did not further reduce  $N_2O$  emissions from the fumigated soil. Indeed,  $N_2O$  emissions from the cycloheximide-treated fumigated soil (Cy) were higher than those measured from the unfumigated soil. Maybe the resistant component of the soil microflora was able to utilise the cycloheximide-N itself, when the competition was lowered by the fumigation.

The trend for the soil respiration rates were quite different from those for  $N_2O$  emissions (Figure 5.9).



**Figure 5.9** - Soil respiration from the unfumigated and the fumigated woodland soil untreated, or treated with substrate and/or fungicide. For the X-axis legend see Fig. 5.8.

$CO_2$  emission rates increased, with first order kinetics, with increasing concentrations of peptone, in both unfumigated and fumigated treatments. The rate of increase was

not significantly different in the two soils ( $\sim 0.1 \mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1} \text{ g}^{-1} \text{ peptone-N}$ ) but the basal level of soil respiration, extrapolated to peptone concentration zero, was about 5 times higher in the fumigated soil than the unfumigated one (34.98 and  $7.53 \mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ , respectively). A similar result was obtained by Powlson and Jenkinson (1976a), who observed that when yeast extract was added to both fumigated and unfumigated soils, the  $\text{CO}_2$  which was derived from the yeast extract was nearly the same for both treatments, whereas the amount of  $\text{CO}_2$  which was evolved from the fumigated control was higher than the  $\text{CO}_2$  evolved from the unfumigated control. Addition of cycloheximide to the peptone-treated unfumigated soil resulted in a slightly lower increasing rate of  $\text{CO}_2$  emissions in function of peptone addition ( $0.087 (\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1} \text{ g}^{-1} \text{ peptone-N})$ ) and a higher basal respiration rate ( $23.65 \mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ ) as compared with the peptone unfumigated treatment. On the contrary, when cycloheximide was added to the fumigated soil treated with peptone, the  $\text{CO}_2$  flux was reduced dramatically.

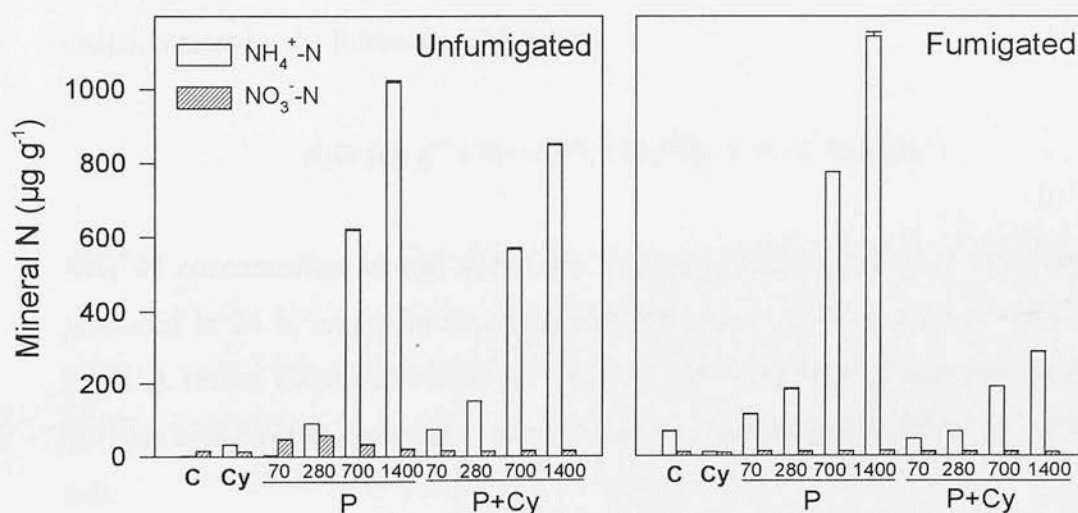
These results suggest that, in the unfumigated soil, microorganisms were mainly utilising peptone as a substrate for respiration, and cycloheximide at a slower rate. Badalucco *et al.* (1994) found that microorganisms were evolving  $\text{CO}_2$  after antibiotics addition to the soil and attributed such flux directly to the respiration of the antibiotic molecules. However, this situation was evident only after 2 days of incubation. After one day, in fact, the opposite was true, i.e. the control respired more. It could be possible that what was really respired after 2 days incubation was part of the microbial population killed by the antibiotics more than antibiotic itself. Landi *et al.* (1993) have indeed concluded that long periods of antibiotics residence in soil result in microbial community changes and non-target effects.

Cycloheximide addition only slightly decreased the respiration rate, suggesting either that bacteria were mainly responsible for the  $\text{CO}_2$  flux, or that the enzymes utilised for respiration of the substrate by the fungal population were not synthesised but pre-existent, so that a protein synthesis inhibitor was not effective. Indeed this last hypothesis could be supported by the results in the fumigated soil. It can be assumed



that during fumigation most of the microbial population was killed (Powlson and Jenkinson, 1976b) and after the fumigation a new population grew. This population was able to utilise peptone almost with the same efficiency as the population in the unfumigated soil. But when cycloheximide was added to the fumigated soil it could have blocked the growth of the fungal population, by blocking protein synthesis, thus reducing substantially the  $\text{CO}_2$  flux after peptone addition, which could then be considered mainly of fungal origin. The remaining small flux of about  $8\text{--}10 \mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$  could have been due instead to the bacterial population, which was not inhibited by cycloheximide. Indeed, this  $\text{CO}_2$  flux was comparable to the flux measured in the fumigated samples treated with cycloheximide alone (Cy). This flux was about one third of the flux in the control fumigated samples (C), as found also by Badalucco *et al.* (1994) in a fumigated forest soil after 24 h incubation with cycloheximide. This could indicate that the inhibited population was of fungal origin while the remaining activity was of bacterial origin and did not contribute to the  $\text{CO}_2$  flux from peptone.

Results for mineral N confirmed several points outlined above (Figure 5.10).



**Figure 5.10** -  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  content in the woodland soil after 24 h incubation. For the X-axis legend see Fig. 5.8.

First of all, from the observation of  $\text{NO}_3^-$ -N, it appeared clear that 7.5 mg of cycloheximide  $\text{g}^{-1}$  were equally effective as fumigation in blocking  $\text{NO}_3^-$  production. Powlson and Jenkinson (1976a), testing the effect of biocidal treatments on nitrification in soil, reported an almost complete block in  $\text{NO}_3^-$  production after fumigation with  $\text{CHCl}_3$ .

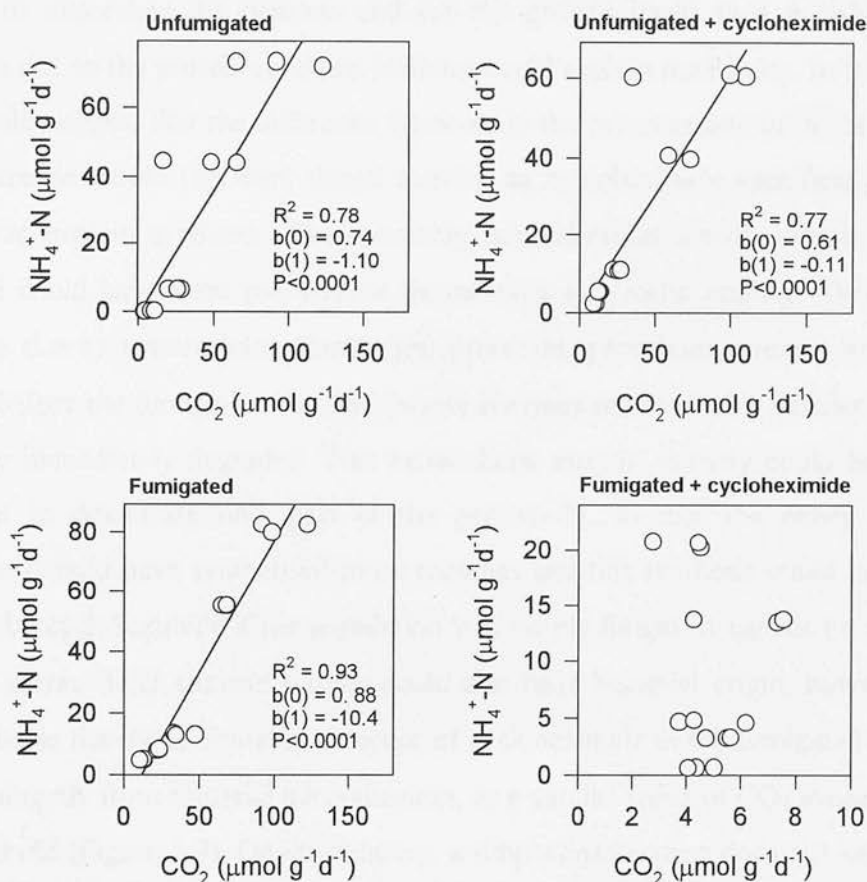
The results from the unfumigated soil treated with increasing concentrations of peptone showed two things: first, that the  $\text{NO}_3^-$  production had a limited maximum rate which did not depend by  $\text{NH}_4^+$  availability; second, that the  $\text{NO}_3^-$  production was inhibited by extremely high concentrations of  $\text{NH}_4^+$ , as indeed  $\text{NO}_3^-$  production at 700 and 1400  $\mu\text{g}$  of peptone-N, was lower than that at 280  $\mu\text{g}$  of peptone-N  $\text{g}^{-1}$ . Nishio and Fujimoto (1990) found a maximum rate of nitrification when the  $\text{NH}_4^+$  concentration was  $< 300 \mu\text{g g}^{-1}$ , after which an inhibitory effect on nitrification was observed for short incubation time (around 30 h). An inhibitory effect of ammonium on nitrification has been observed by other authors and attributed to toxic concentrations of  $\text{NH}_3$  at high pH (Broadbent *et al.*, 1957), to an increase in salt content (Harada and Kai, 1968), or to an increase in osmotic pressure (Darrah *et al.*, 1986).

$\text{NO}_3^-$  production during 24 h was significantly correlated ( $P < 0.0001$ ) with  $\text{N}_2\text{O}$  emission rates by the following relationship:

$$\text{N}_2\text{O} (\text{ng g}^{-1} \text{h}^{-1}) = -0.36 + (0.0763 \times \text{NO}_3^- \text{-N} (\mu\text{g g}^{-1}))$$

$\text{NH}_4^+$ -N concentration in soil after 24 h was significantly correlated with the  $\text{CO}_2$  produced in 24 h, except in the fumigated soil treated with cycloheximide (Figure 5.11). A similar linear correlation ( $R = 0.973$ ) was found by Powlson and Jenkinson (1976a) between  $\text{CO}_2$  flux and N mineralization in both a fumigated and an untreated soil.

The fumigated soil treated with peptone showed the highest rate of  $\text{NH}_4^+$  production in function of the respiration (Figure 5.11). The lowest rate was found in the unfumigated soil treated with cycloheximide.



**Figure 5.11** - Relationship between  $\text{CO}_2$  flux and  $\text{NH}_4^+\text{-N}$  found in the soil, measured after 24h incubation in the woodland soil treated with different concentrations of peptone (see § 5.2.2). Soil was either unfumigated or fumigated and treated with or without cycloheximide.

Comparing the peptone-treated fumigated soil with the fumigated soil treated with peptone and cycloheximide, there was a big difference in the mineralized peptone-N. In the former treatment, the growing microbial population was still able to mineralize peptone more efficiently than in the unfumigated soil, but this ability was drastically reduced by cycloheximide in the latter treatment. If such a dramatic decrease was induced by a lethal effect of cycloheximide on the overall microbial population this would also have been observed in the unfumigated soil. This was not the case. Instead

if it is assumed that the growing population was synthesising *de novo* the enzymes needed to mineralize the peptone and for the growth itself, then a reduction of synthesis due to the protein synthesis inhibitor could explain the results. In this case it also would suggest that the difference observed in the presence and in the absence of cycloheximide would represent fungal activity, as cycloheximide specifically inhibits eukaryotic protein synthesis. The remaining mineralization activity which was not inhibited could have been pre-existent deaminating enzymatic activity. This activity could be due to extracellular deaminases, proteases, peptidases, present in the soil already before the fumigation, as cytoplasmic enzymes released after cellular lysis are generally immediately degraded. This extracellular enzyme activity could have been sufficient to deaminate only part of the peptone-N, so that the newly growing population could have synthesised more enzymes and this synthesis could have been blocked by cycloheximide if the population was mainly fungal. It cannot be excluded that the extracellular enzyme activity could also have bacterial origin, however it is less probable that  $\text{NH}_4^+$  found in presence of cycloheximide in the fumigated soil was derived directly from bacterial mineralization, as a parallel trend of  $\text{CO}_2$  evolution was not observed (Figure 5.9). On the contrary, a simple deamination does not necessarily involve a complete degradation of the protein with consequent release of  $\text{CO}_2$ .

In conclusion the results presented suggest that  $\text{N}_2\text{O}$  emissions deriving from peptone addition were of fungal origin, as was most of the  $\text{CO}_2$  derived from peptone mineralization. Also the biggest part of the  $\text{NH}_4^+$  mineralized from peptone seemed to be due to fungal activity, though at low concentrations of peptone part of the  $\text{NH}_4^+$  seemed to derive from extracellular pre-existent microbial enzyme activity.

### **5.3 EFFECT OF STREPTOMYCIN AND CYCLOHEXIMIDE ON PEPTONE-N MINERALIZATION AND $\text{N}_2\text{O}$ PRODUCTION**

The following experiment was set up to evaluate the effect of an inhibitor of bacterial protein synthesis on peptone-induced  $\text{N}_2\text{O}$  emissions and to confirm the results obtained in § 5.2, which suggested that bacteria could have contributed only in a

minor way to the mineralization of peptone-N, but had a main role in the mineralization of the cycloheximide molecule

For this purpose, streptomycin was used at low, but increasing, concentrations in order to try to avoid non-target effects (Ingham and Coleman 1984; Landi *et al.*, 1993, Badalucco *et al.*, 1994), and also to provide better evidence of any eventual inhibition activity of the molecule. Over a range of streptomycin concentrations which are not bactericidal or fungicidal, increasing the concentration of streptomycin should improve the efficiency of the inhibition, offering further confirmation about the inhibited population through the increasing inhibition.

5.3.2 EXPERIMENTAL DESIGN

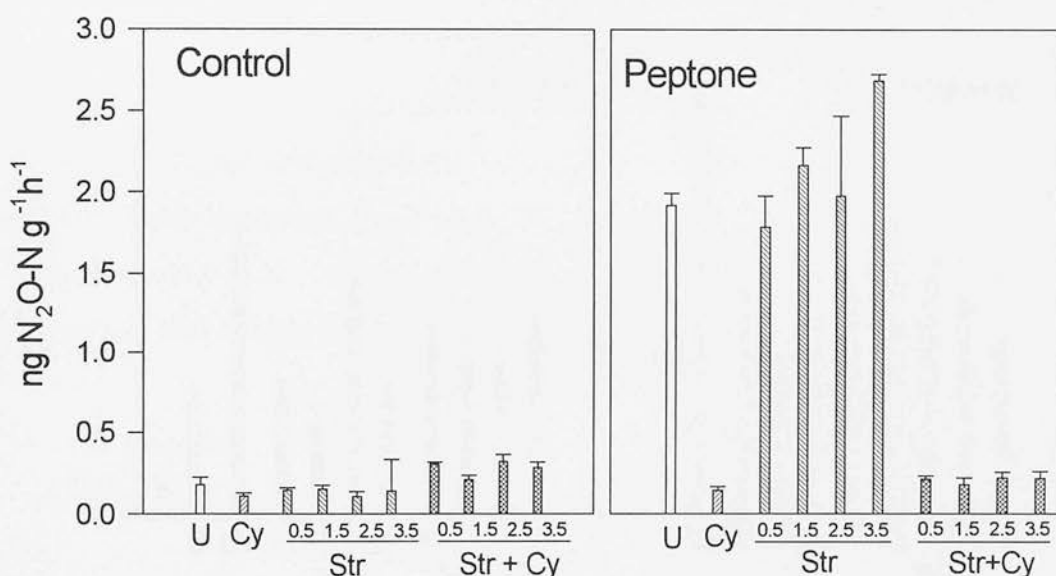
Soil was sampled and sieved as described before (§ 5.1.2). The next evening it was pre-incubated (12h) with cycloheximide and/or streptomycin as reported in Table 5.2, as previously described (§ 4.2.2).

Table 5.2 - Quantity of antibiotics added to the woodland soil.

Samples label	Cycloheximide <i>mg g<sup>-1</sup> dry soil</i>	Streptomycin <i>mg g<sup>-1</sup> dry soil</i>
U (Untreated)	-	-
Cy (cycloheximide only)	7.5	-
Str (streptomycin only)	-	0.5 or 1.5 or 2.5 or 3.5
Str + Cy (both antibiotics)	7.5	0.5 or 1.5 or 2.5 or 3.5

After 12 h of pre-incubation, half of the samples were mixed with peptone (70 µg N g<sup>-1</sup> dry soil). Water content was adjusted to 60% of WFPS. Each treatment was done on three replicates of 25 g each. Samples were incubated in 1 l air-tight jars and incubated for 24 h at 25°C, after which two sequential gas samples, of 2 ml each, were withdrawn with greased-glass syringes for N<sub>2</sub>O and CO<sub>2</sub> analysis and soil was extracted for mineral N analysis (for details on routine methods see chapter 2).

As already demonstrated above,  $\text{N}_2\text{O}$  emissions were significantly higher (about 10-fold) in the samples treated with peptone as compared with the control (Figure 5.12). In the control, the addition of antibiotics had no significant effect (positive or negative) on  $\text{N}_2\text{O}$  fluxes.



**Figure 5.12** -  $\text{N}_2\text{O}$  emission rates from the woodland soil treated with or without peptone and a combination of antibiotics. For x-axis legend see Table 5.2.

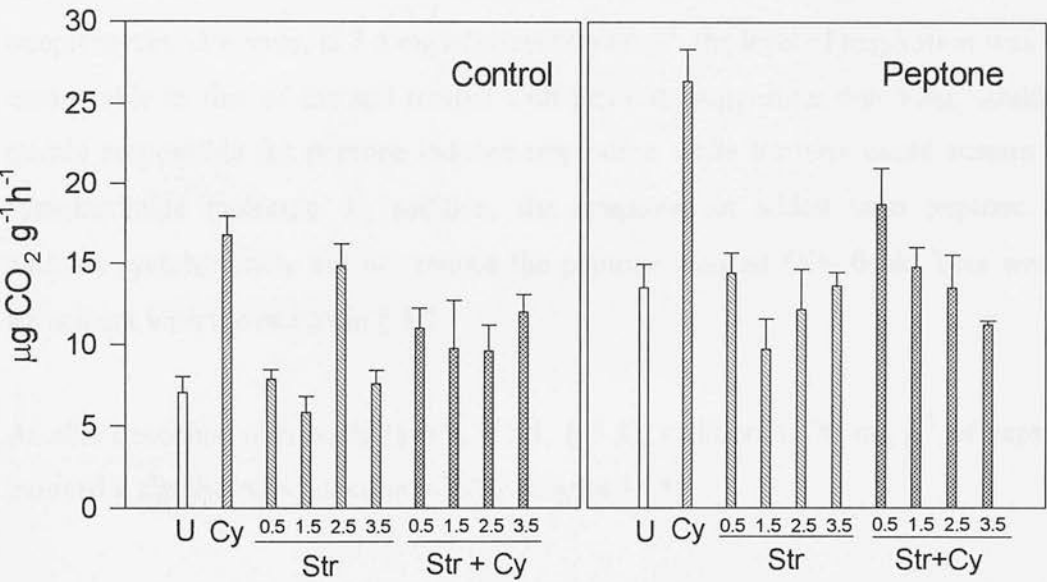
In the peptone treated soil, addition of 7.5 mg of cycloheximide  $\text{g}^{-1}$  reduced  $\text{N}_2\text{O}$  emissions to the control level (around  $0.2 \text{ ng N g}^{-1} \text{h}^{-1}$ ). In contrast, not only was streptomycin sulphate ineffective in reducing  $\text{N}_2\text{O}$  emissions from the peptone treated samples but 3.5 mg of streptomycin  $\text{g}^{-1}$  induced a further increase in  $\text{N}_2\text{O}$  emissions. This stimulation of  $\text{N}_2\text{O}$  production was not observed when streptomycin was added together with cycloheximide.

Soil respiration was enhanced in the presence of antibiotics, alone or in combination (Figure 5.13). In the control, the addition of cycloheximide increased soil respiration by about  $10 \mu\text{g CO}_2 \text{ g}^{-1} \text{h}^{-1}$ . Contrasting results have been found when cycloheximide



is added to the soil and the CO<sub>2</sub> flux varied greatly depending on the incubation time (Landi *et al.*, 1993; Badalucco *et al.*, 1994).

The CO<sub>2</sub> flux in the presence of streptomycin was not different from the control, whereas streptomycin significantly reduced the CO<sub>2</sub> flux induced by cycloheximide addition (Figure 5.13).



**Figure 5.13** - Soil respiration rates from the woodland soil treated with or without peptone and with different concentrations of antibiotics. For x-axis legend see Table 5.2.

Peptone addition stimulated CO<sub>2</sub> production (U), though the flux was not greater than the flux induced by cycloheximide addition in the control. When peptone was added together with cycloheximide the CO<sub>2</sub> production increased significantly (Figure 5.13). Again, streptomycin did not significantly reduce the peptone induced CO<sub>2</sub> flux but drastically reduced the CO<sub>2</sub> flux induced by cycloheximide. This last reduction significantly increased with increasing concentrations of streptomycin.

Addition of both antibiotics to the soil has been found in some cases to increase soil respiration and in some other cases to decrease it (Landi *et al.*, 1993; Badalucco *et*

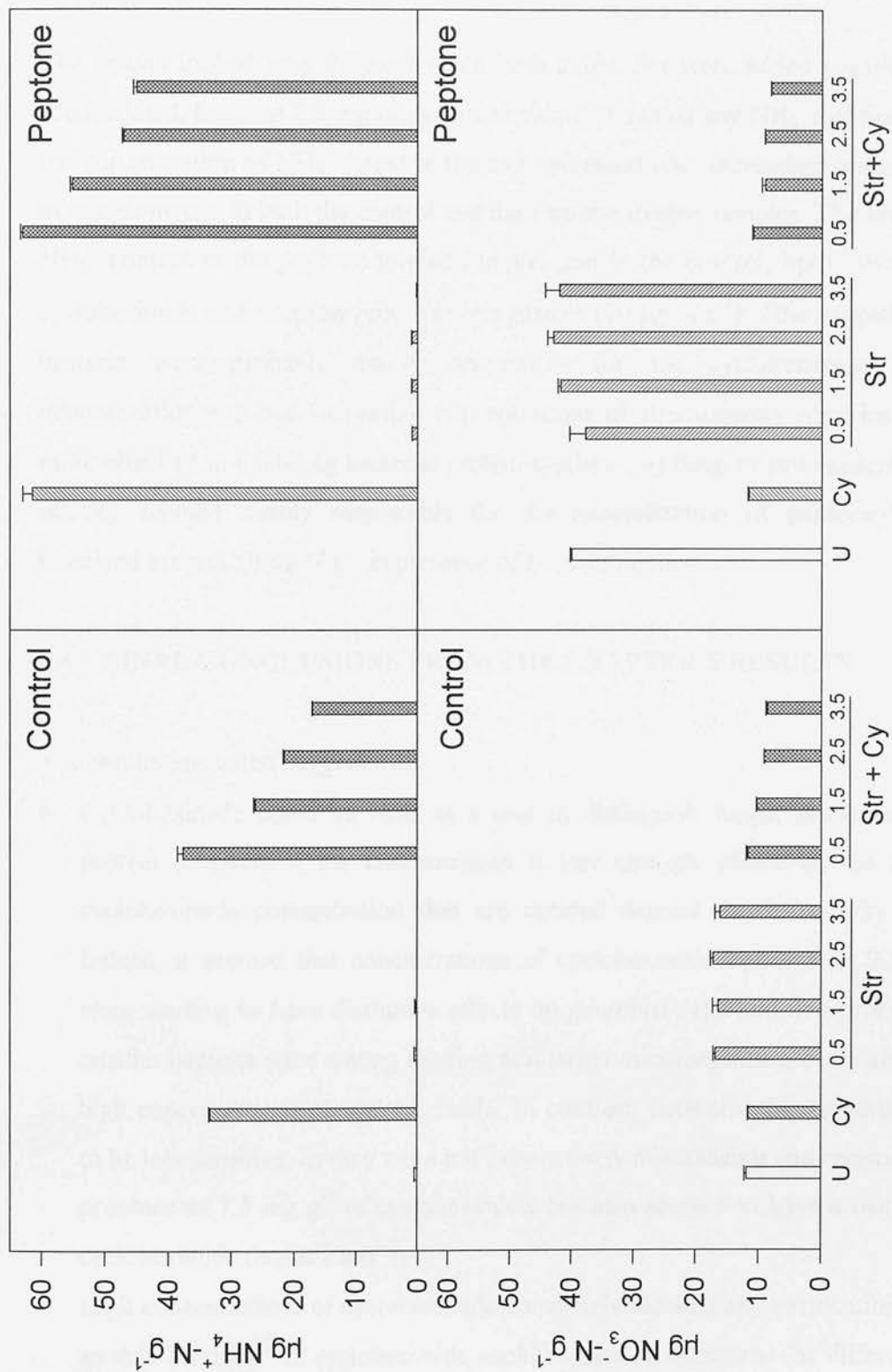
*al.*, 1994). In the presence of an additional source of C, Anderson and Domsch (1973) found a net decrease of soil respiration in the presence of both antibiotics.

In the present case, cycloheximide stimulated soil respiration, probably stimulating some specialized bacterial activity, which was further enhanced by the eliminated competition with fungi. Indeed, in both the control and the peptone treated soils, when the bactericide was also added, the extra flush of CO<sub>2</sub> deriving from the cycloheximide addition was increasingly reduced with increasing concentration of streptomycin. However, at 3.5 mg of streptomycin g<sup>-1</sup>, the level of respiration was still comparable to that of the soil treated with peptone, suggesting that fungi could be mainly responsible for peptone induced-respiration while bacteria could respire the cycloheximide molecule. In addition, the streptomycin added with peptone but without cycloheximide did not reduce the peptone-induced CO<sub>2</sub> flush. This was in agreement with the results in § 5.2.

As also described previously (§ 4.2, § 5.1, § 5.2), addition of 70 mg g<sup>-1</sup> of peptone induced a significant production of NO<sub>3</sub><sup>-</sup> (Figure 5.14).

When cycloheximide was added, the NO<sub>3</sub><sup>-</sup> production was kept down to the control level, while NH<sub>4</sub><sup>+</sup> accumulated in both the control and in the peptone treated samples. As found previously (§ 4.2.3), the NO<sub>3</sub><sup>-</sup> derived from peptone was around 30 µg N g<sup>-1</sup>, while the mineralized N was about 50 % of the added N. The NH<sub>4</sub><sup>+</sup> derived from the addition of cycloheximide was around 30 µg N g<sup>-1</sup> at the end of the incubation period.

Streptomycin did not induce any NH<sub>4</sub><sup>+</sup> accumulation in either the peptone or the control and was not effective in reducing the peptone induced NO<sub>3</sub><sup>-</sup> production. In the control, it slightly enhanced the NO<sub>3</sub><sup>-</sup> production, indicating that some N was probably mineralized from the streptomycin molecule and nitrified.



**Figure 5.14** -  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  measured in the woodland soil after 24 h incubation. For the x-axis legend see Table 5.2.

As streptomycin was not blocking nitrification (either peptone induced or induced by the molecule itself), it seemed that fungi were more likely than bacteria to have been responsible for the  $\text{NO}_3^-$  produced.

The results looked very different when both antibiotics were added together.  $\text{NH}_4^+$  accumulated, because  $7.5 \text{ mg}$  of cycloheximide  $\text{g}^{-1}$  blocked any  $\text{NH}_4^+$  nitrification, but the concentration of  $\text{NH}_4^+$  found in the soil decreased with increasing concentrations of streptomycin, in both the control and the peptone treated samples. The decrease of  $\text{NH}_4^+$  content in the peptone treated samples and in the control, both treated with cycloheximide and streptomycin, was comparable ( $20 \text{ } \mu\text{g N g}^{-1}$ ). This suggests that: i) bacteria were probably mainly responsible for the cycloheximide molecule mineralization and that increasing concentrations of streptomycin were increasingly more effective in inhibiting bacterial protein synthesis; ii) fungi or pre-existent enzyme activity seemed mainly responsible for the mineralization of peptone-N which remained around  $30 \text{ } \mu\text{g N g}^{-1}$  in presence of both antibiotics.

## 5.4 FINAL CONCLUSIONS FROM THE CHAPTER 5 RESULTS

The results presented suggest that:

- Cycloheximide could be used as a tool to distinguish fungal substrate-induced protein synthesis if the concentration is low enough. However, the limits of cycloheximide concentration that are optimal depend on the activity studied. Indeed, it seemed that concentrations of cycloheximide higher than  $2.5 \text{ mg g}^{-1}$  were starting to have disruptive effects on microbial cells and it is probable that nitrifier bacteria were among the first non-target microorganisms to be affected by high concentrations of cycloheximide. In contrast, heterotrophic bacteria seemed to be less sensitive, as they were not only actively mineralizing and respiring in the presence of  $7.5 \text{ mg g}^{-1}$  of cycloheximide, but also seemed to have a main role in cycloheximide degradation.
- High concentrations of cycloheximide completely blocked any nitrification activity so that  $7.5 \text{ mg g}^{-1}$  of cycloheximide could be used to determine (by difference) the

total N which was nitrified after a substrate addition. This was proved to be true in the woodland soil and in the arable soil (Appendix III); however, to generalise this conclusion more soils should be tested and measurements of gross rates of nitrification in the presence of cycloheximide should be made (see chapter 6).

- Fungi seemed to be mainly responsible for peptone-induced  $\text{N}_2\text{O}$  emissions as well as  $\text{NO}_3^-$  production. However, the main mechanism of  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  production seemed to involve ammonia monooxygenase synthesis and activity. This is in disagreement with general views on fungal nitrification though data on this topic are very scarce.
- Fungi seemed also to be mainly responsible for peptone mineralization, while they did not seem to contribute significantly to cycloheximide degradation.

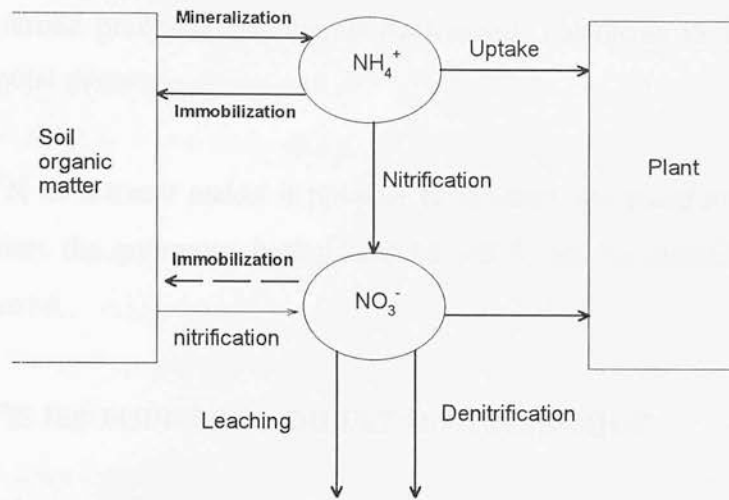
## USE OF $^{15}\text{N}$ ISOTOPE TECHNIQUES TO STUDY THE EFFECTS OF ANTIBIOTICS ON SOIL N MINERALIZATION AND NITRIFICATION

### 6.1 THE $^{15}\text{N}$ TRACER TECHNIQUE IN SOIL STUDIES: AN INTRODUCTION

The isotope  $^{15}\text{N}$  was discovered in 1929 by Naude. It, and the more abundant  $^{14}\text{N}$  are stable and occur in the air in a ratio of 272:1; in the normal terminology  $^{15}\text{N}$  has a *natural abundance of 0.3663 atom % (1/273)*. Several unstable (radioactive) isotopes are now also known:  $^{13}\text{N}$ ,  $^{12}\text{N}$ ,  $^{16}\text{N}$  and  $^{17}\text{N}$ , of which  $^{13}\text{N}$  has the longest half life, 9.97 minutes. Because  $^{15}\text{N}$  is stable, not radioactive and has such a low natural abundance against the enormous background of  $^{14}\text{N}$ , it is commonly used as a tracer to follow the fate of N compounds nature in complex systems such as soil.

An apparently simple N cycle in the soil/plant system involves 6 processes and four nitrogen pools (Figure 6.1), where “pool” is defined as a fraction of nitrogen which can be characterized by chemical analysis.





**Figure 6.1** - Soil/plant nitrogen cycle.

Due to the complexity of soil nitrogen transformations, the simple measurement of a pool's size over time does not often give precise information on the flows of nitrogen from one pool to another. The ammonium pool results, for example, from a dynamic equilibrium between processes which consume ammonium such as immobilization, plant uptake, volatilization, nitrification, and processes which produce ammonium such as mineralization or ammonification. A measure of the mineralization process becomes then quite complicated if we follow only the ammonium pool in soil. Non-isotopic methods can generally give information only on net mineralization ( $\Delta M$ ) through the nitrogen balance equation:

$$\Delta M = \Delta \text{NH}_4^+ + \Delta \text{NO}_3^- + \Delta \text{ plant} + \text{loss (leaching or gas)}$$

Even where high precision is possible, the method requires a big effort to measure the N present in all the pools considered. When laboratory studies are performed the experimental conditions can be simplified. Several methods are available such as aerobic incubations where soil are either leached (Stanford and Smith, 1972) or incubated in closed containers (Keeney, 1982) and anaerobic incubations (Waring and Bremner, 1964). In any case the problem of measuring gross mineralization remains.

The same problems are found to measure gross nitrification rates as it is necessary to measure the nitrate produced and the nitrate leached, volatilized or immobilized in plant or microbial tissue.

The use of  $^{15}\text{N}$  as a tracer makes it possible to measure the transformations of the added N against the enormous background of soil N and to quantify directly the process of interest.

## 6.2 THE $^{15}\text{N}$ ISOTOPE POOL DILUTION TECHNIQUE

The  $^{15}\text{N}$  pool dilution technique has been widely used to quantify gross N transformation rates in soil (Kirkham and Bartholomew 1954, 1955; Barraclough, 1985; Nishio *et al.*, 1985; Myrold and Tiedje, 1986; Barraclough, 1991; Barraclough and Puri, 1995). The method is based on the principle that when an N pool is enriched with  $^{15}\text{N}$ , an input of unlabelled N into the N pool lowers the  $^{15}\text{N}$  abundance, whereas any process which removes N from the same pool does it in proportion to the amounts of  $^{14}\text{N}$  and  $^{15}\text{N}$  present and consequently will not alter the  $^{15}\text{N}$  abundance of the pool. This assumption of proportional exploitation can be accepted when the  $^{15}\text{N}$  enrichment is near natural abundance. Under these conditions, a process which introduces N at natural abundance in the pool (e.g. mineralization -  $\text{NH}_4^+$  pool or nitrification -  $\text{NO}_3^-$  pool) will cause a decline in the  $^{15}\text{N}$  pool abundance. This decline will be faster, the greater is the rate of influx of unlabelled N and the rate of removal of N ( $^{14}\text{N}+^{15}\text{N}$ ) from the pool.

There are two approaches for the determination of gross processes: those based on analytical equations (Kirkham and Bartholomew 1954, 1955; Barraclough *et al.*, 1985; Nishio *et al.*, 1985; Wessel and Tietema, 1992), which relate the change in  $^{15}\text{N}$  abundance of the labelled N pool to the gross rate of the process; and simulation models, which allow the determination of rates of mineralization, immobilization or nitrification by searching for parameters that result in pool  $^{15}\text{N}$  abundances equal to

those measured by experimental procedure (Paul and Juma, 1981; Bjarnason, 1988; Barraclough and Puri, 1995).

The former method is simpler to use but it is restricted in its applicability by the validity of the assumptions which need to be made to derive the analytical equations, which are:

- (a) the studied processes do not discriminate between  $^{14}\text{N}$  and  $^{15}\text{N}$ ;
- (b) added  $^{15}\text{N}$  is mixed homogeneously with soil so that labelled and unlabelled N are used in proportion to the relative amount present;
- (c) labelled N immobilized over the experimental period is not remineralized;
- (d) gross transformation rates remain constant throughout the measurement period.

The two last assumptions are the most restrictive.

Kirkham and Bartholomew (1954, 1955) presented a set of analytical equations valid for experiments with high  $^{15}\text{N}$  enrichments and short duration, assuming no recycling of the immobilized  $^{15}\text{N}$ . Blackburn (1979) extended the applicability of these equations to experiments with low addition of  $^{15}\text{N}$ . In recent years a number of workers extended and simplified the equations (Barraclough *et al.* 1985; Nishio *et al.*, 1985; Myrold and Tiedje, 1986; Barraclough, 1991). To calculate gross rates of mineralization and nitrification, using the pool dilution, the following set of equations has been proposed by Barraclough *et al.* (1985).

Gross mineralization rate is given by:

$$A_t^* = A_0^* / (1 + \theta t / A_0)^{m/\theta} \quad (1)$$

where  $A$  is the size of the ammonium pool ( $^{14}\text{N} + ^{15}\text{N}$ ),  $m$  is the mineralization rate,  $\theta$  is the rate of change of the ammonium pool,  $*$  is the atom % excess.

The rate at which the ammonium pool changes size is given by:

$$\theta = m - i - l - n - u_a \dots\dots\dots(2)$$

where  $i$  is the immobilization,  $l$  is any loss from the system,  $n$  is the nitrification and  $u_a$  is plant uptake. In experimental terms:

$$\theta = \frac{(A_t - A_0)}{t} \quad (3)$$

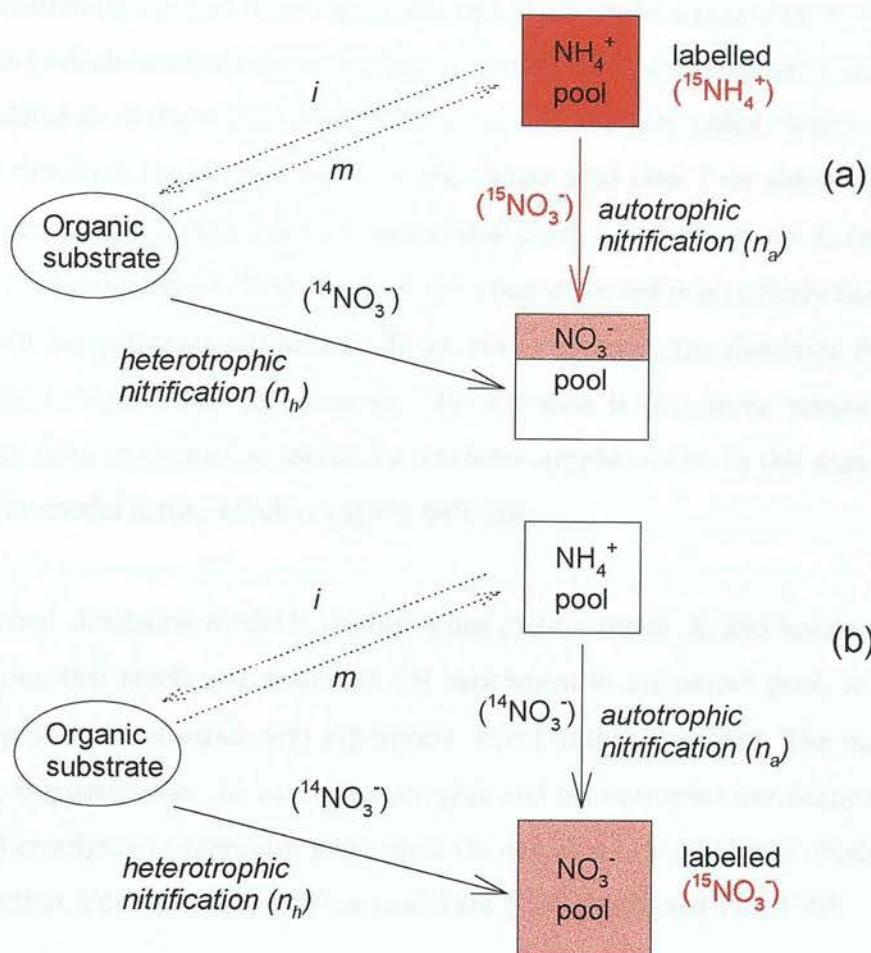
The same type of equation can be used to calculate the gross rate of nitrification:

$$N_t^* = N_0^* / (1 + \theta t / N_0) n / \theta \quad (4)$$

where  $N$  is the size of the nitrate pool ( $^{14}\text{N} + ^{15}\text{N}$ ),  $n$  is the nitrification rate,  $\theta$  is the rate of change of the nitrate pool,  $*$  is the atomic % excess.

The gross nitrification rate, determined by pool dilution, gives the rate of nitrate production by the combined autotrophic and heterotrophic routes ( $n_a + n_h$ ). In contrast, nitrification rate calculated with pool enrichment depends only on the autotrophic route (Figure 6.2).

Thus, in an experiment where half of the plots receive labelled ammonium and unlabelled nitrate and half receive the reverse, it is possible to calculate the total gross rates of nitrification ( $n_a + n_h$ ) from the pool dilution experiment and the gross rate of autotrophic nitrification from the pool enrichment experiment and obtain as a difference the gross rate of heterotrophic nitrification. However, interpreting pool enrichment data is quite difficult because  $^{15}\text{N}$  abundance of both ammonium and nitrate changes over time so that analytical solutions are only an approximation (Nishio *et al.*, 1985; Wessel and Tietema, 1992; Barraclough and Puri, 1995). Because of this limitation, interpreting enrichments experiment in terms of rates of nitrogen transformation requires simulation modelling (Barraclough and Puri, 1995).



**Figure 6.2** - Diagram representing the input or the dilution of <sup>15</sup>NO<sub>3</sub><sup>-</sup> in the nitrate pool in an enrichment experiment (a) and a pool dilution experiment (b), respectively, via autotrophic (n<sub>a</sub>) and/or heterotrophic (n<sub>h</sub>) nitrification route. (m: mineralization; i: immobilization).

### Simulation modelling

Two simulation models have been used to calculate rates of autotrophic and heterotrophic nitrification for the experiments presented in this chapter. The method is reported by Barraclough and Puri (1995). The first model simulates the flow of N and <sup>15</sup>N between an initially labelled ammonium pool and a nitrate pool receiving the



size and the  $^{15}\text{N}$  atom % of the ammonium and nitrate pools at  $t=0$ , the gross rate of mineralization (calculated from Eq. 1), the rate at which the ammonium pool changes size (from which is calculated the decline of  $^{15}\text{NH}_4^+$ ) and the rate of nitrification (Eq. 4) calculated from the parallel experiment with  $^{15}\text{NO}_3^-$  initially added. When the model runs, it simulates the increase of  $^{15}\text{N}$  in the nitrate pool over time assuming that *all nitrate production comes from the ammonium pool, i.e. there is not heterotrophic route*. If the model gives  $^{15}\text{NO}_3^-$  very close to that observed it is unlikely that there is significant heterotrophic nitrification. If, on the other hand, the simulated final value of  $^{15}\text{NO}_3^-$  is higher than the observed, the inference is that some nitrate is being produced from an unlabelled source, i.e. via heterotrophic route. In this case a second simulation model is run, which is slightly different.

This second simulation model hunts for values of autotrophic  $n_a$  and heterotrophic  $n_h$  nitrification that result in a simulated  $^{15}\text{N}$  enrichment in the nitrate pool, in the final sampling of the pool enrichment experiment, equal to that measured. The model runs with the constraint that the sum of autotrophic and heterotrophic nitrification rates in the pool enrichment experiment must equal the overall nitrification rate obtained from pool dilution. For more details of the model see Barraclough and Puri 1995.

## 6.3 THE ANALYTICAL METHODS

The experimental method can be divided into three phases: i) the preparation and incubation of the samples; ii) the extraction of the  $^{14}\text{N}$  and  $^{15}\text{N}$  from the mineral pool of the soil; iii) the measurements of the extracted N and the calculations.

### 6.3.1 SAMPLES PREPARATION AND INCUBATION

- Soil is sieved (2 mm) after sampling to allow a more even distribution of the added  $^{15}\text{N}$ .
- Soil (40 g) is put in a jar and  $^{15}\text{N}$  is added in form of solution of an N salt, usually  $(^{15}\text{NH}_4)_2\text{SO}_4$  or  $\text{K}^{15}\text{NO}_3$ , with a final  $^{15}\text{N}$  abundance around 10%. Half of the



samples receive labelled ammonium sulphate and unlabelled potassium nitrate, the other half receive the reverse. For both treatments two blocks of samples are prepared, one to be extracted at  $t = 0$  and another at  $t = t$ .

- The jars are closed in order to avoid any water loss and are incubated at 25°C in a thermostated room. The length of the incubation can vary, depending on the purpose of the experiment, however it is important not to go further than a week incubation (Powelson and Barraclough, 1993) in order to avoid processes of remineralization of the N immobilized in the microbial biomass, and hence further complications in the measurements of the  $^{14}\text{N}$  and  $^{15}\text{N}$  pools. In the following experiment we have used an incubation time of 24 h, in order to have the same incubation period used in the experiment done with non isotopic techniques.
- Immediately after the samples " $t = t$ " are incubated, the samples " $t = 0$ " are extracted (see next paragraph) for the measurements of the size and abundance of the mineral-N pools.

### 6.3.2 EXTRACTION OF LABELLED MINERAL N

Conventional methods for  $^{15}\text{N}$  extraction and analysis are tedious and require labour-intensive sample preparation and quite a large sample mass (Buresh *et al.*, 1982; Pruden *et al.*, 1985). Diffusion is a recognized alternative to steam distillation in the measurement of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  in soil and has been used to concentrate sample extracts before  $^{15}\text{N}$  analysis (Keeney and Nelson, 1982; Adamsen and Reeder, 1983; MacKown *et al.*, 1987). This technique has been recently improved to reduce cross contamination, to reduce the sample volume needed and to reduce the preparation time (Brookes *et al.*, 1989; Mulvaney, 1993). The procedure, slightly modified from Brooks *et al.* (1989), is very simple, fast and has been proved to work without problems if the precautions to avoid cross contamination are taken:

- 20 g of soil are extracted with 100 ml of 1 M KCl as described in § 2.7.1. After soil has been shaken for 1 h with extractant, the slurry is filtered using glass fibre filter paper. It is important to use these special filters instead than normal filter paper which could contain small quantities of N.

- Part of the extract is used to measure the concentration of total  $\text{NH}_4^+$  ( $^{14}\text{NH}_4^+$  and  $^{15}\text{NH}_4^+$ ) and the total  $\text{NO}_3^-$  ( $^{14}\text{NO}_3^-$  and  $^{15}\text{NO}_3^-$ ) extracted from the soil, using standard techniques for the measurements of mineral N (see § 2.7.1).
- An other part of the soil 1 M KCl extract is used for the extraction of  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$  using the following procedure:
  1. 20 - 60 ml of extract (which should contain no more than 20-100  $\mu\text{g}$  inorganic N at  $< 30\%$   $^{15}\text{N}$ ) are placed in a 500 ml Erlenmeyer flask;
  2. MgO (preheated to 1000 °C for 2 h) is added (about 0.2 g scoop) to buffer the solution to about pH 10.5. This allows the  $\text{NH}_4^+$  present into solution to pass in the  $\text{NH}_3$  form and volatilize. For this reason the lid is immediately and tightly closed. The lid is provided with a stainless steel wire on which is inserted a 4 mm disc of glass-fiber filter acidified with 10  $\mu\text{l}$  of 2.5 M  $\text{KHSO}_4$  (which can trap up to 350  $\mu\text{m}$  N);
  3. The containers are left for 24 h on a shaker and the  $\text{NH}_3$  which volatilizes is trapped on the acidified paper. The paper is then collected and left to dry. In the moment that the paper is collected great care must be used in order to avoid any cross-contamination. The disc is gently pushed out of the wire with a little piece of wire or a paper clip and afterwards it is placed into a rack with several numbered wells and the rack is immediately put into a desiccator over  $\text{CaSO}_4$ ;
  4. The flasks are left open overnight on the shaker in order to leave any residual  $\text{NH}_3$  to diffuse, to avoid cross contamination, as what is important is the  $^{15}\text{N}$  % abundance and consequently it is not important to recover all the  $\text{NH}_4^+$  present in solution. Since combustion mass spectrometry requires less than 100  $\mu\text{g}$  N, it is not necessary to use 100% of the trapping capacity of the filter;
  5. The next day finely ground Devarda's alloy (0.5 g) is added to the flasks in order to reduce  $\text{NO}_3^-$  to  $\text{NH}_4^+$ , another acidified filter is added to the lid, the flask is closed and put on the shaker for 24 hours. As the solution is still basic (due to the presence of MgO), all the  $\text{NO}_3^-$  reduced to  $\text{NH}_4^+$

volatilizes as  $\text{NH}_3$  and is trapped on the second paper disk. After 24 h the disks are collected and put to dry as described in point 3;

6. After having dried, discs are put in Sn capsules, which are crimped with tweezers and introduced into an automatic N analyser coupled to an isotope ratio mass spectrometer, for  $^{15}\text{N}$  analysis (see below).
  7. It is important to diffuse also a blank with the sole KCl solution used to extract the soil.
- The same procedure is applied to the sample extracted at  $t=0$  and  $t=t$ .

### 6.3.3 ANALYSIS OF THE SAMPLES

Although several techniques are available for N isotope analysis, isotope ratio mass spectrometry is the method which provides the biggest precision (Bremner 1965; Buresh *et al.*, 1982; Robinson and Smith, 1991). This advantage, together with the high sensitivity reached by the most recent instruments, makes mass spectrometry the method of choice for quantitative determination in isotopic analysis.

Mass spectrometers can be interfaced with an automatic N/C analyzer (ANCA) (Otsuki *et al.*, 1983; Harris and Paul, 1989; Craswell and Eskew, 1991; Jensen, 1991). Such a system is generally referred as ANCA-MS.

In the present experiments, done at Reading University in collaboration with Dr D. Barraclough, the  $^{15}\text{N}:^{14}\text{N}$  ratios in the ammonium and nitrate fractions were determined on a VG Micromass 622 mass spectrometer linked to a Europa Scientific RoboPrep combustion analyser and referenced against IAEA  $^{15}\text{N}$  quality control standard 305. No spiking was employed as reliable isotope ratios were possible on 20  $\mu\text{g}$  N using this system.

## 6.4 EFFECTS OF HIGH CONCENTRATIONS OF CYCLOHEXIMIDE AND STREPTOMYCIN ON GROSS RATES OF MINERALIZATION AND NITRIFICATION

### 6.4.1 INTRODUCTION

In the following experiment the isotope dilution and enrichment techniques were used to evaluate the effect of high concentrations of cycloheximide ( $7.5 \text{ mg g}^{-1}$ ) and streptomycin ( $3.5 \text{ mg g}^{-1}$ ) on gross rates of mineralization and nitrification, as previous experiments (see chapter 4 and 5) showed that concentrations of cycloheximide higher than  $2.5 \text{ mg g}^{-1}$  soil had a disruptive effect on nitrifiers population, while mineralization increased for increasing addition of cycloheximide up to  $7.5 \text{ mg cycloheximide g}^{-1}$ . On the contrary,  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  producers seemed insensitive to streptomycin addition.

### 6.4.2 EXPERIMENTAL DESIGN

Soil was sampled from the top 20 cm of the woodland soil sieved (2 mm) and stored at  $15^\circ\text{C}$  in a thermostated room. The next day soil was treated with no antibiotics, or with  $7.5 \text{ mg}$  of cycloheximide  $\text{g}^{-1}$ , or with  $3.5 \text{ mg}$  of streptomycin sulphate  $\text{g}^{-1}$ , or with both  $7.5 \text{ mg}$  of cycloheximide  $\text{g}^{-1}$  and  $3.5 \text{ mg}$  of streptomycin sulphate  $\text{g}^{-1}$ , added to the soil as previously described (§ 4.2.2). Water content was adjusted to reach 60% WFPS. After 10 h half of the samples received  $70 \text{ }\mu\text{g}$  of peptone-N  $\text{g}^{-1}$ . Immediately after peptone addition, the samples were treated with  $^{15}\text{N}$ . Each treatment was divided into two blocks (done on triplicate): one receiving a solution containing  $(^{15}\text{NH}_4)_2\text{SO}_4 + \text{K}^{14}\text{NO}_3$ ; and another one receiving a solution containing  $(^{14}\text{NH}_4)_2\text{SO}_4 + \text{K}^{15}\text{NO}_3$ . Both the solutions contained  $200 \text{ }\mu\text{g N ml}^{-1}$  and the  $^{15}\text{N}$  used for the solution was 10 atom % enrichment. Soil samples (40 g) were weighed in plastic bottles and 2 ml of  $^{15}\text{N}$  solution ( $10 \text{ }\mu\text{g N g}^{-1}$  soil) were added in little drops, mixing thoroughly, in order

to distribute the  $^{15}\text{N}$  as well as possible, then the bottles were firmly capped to avoid water losses.

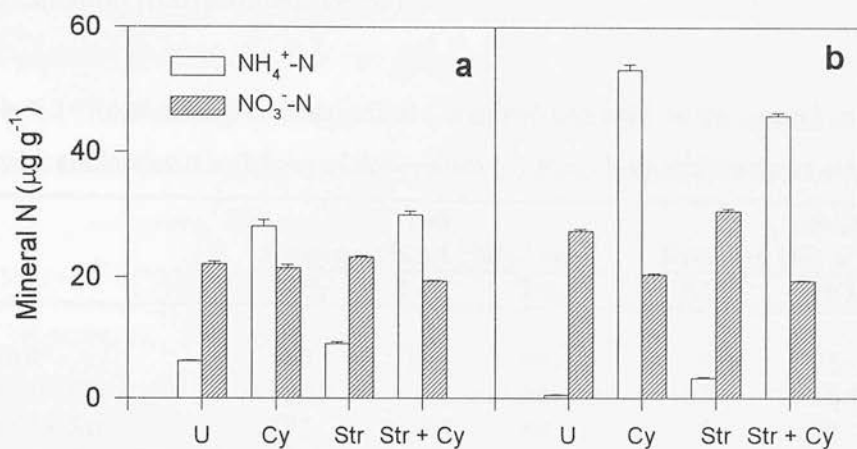
One set of samples was incubated at  $25^{\circ}\text{C}$ , while another set was immediately extracted after the preparation to measure the size and  $^{15}\text{N}$  abundance of the mineral N pools at *time zero*. After 24 h also the second set of samples was extracted. Analysis of mineral N on the soil extraction was performed as described in § 2.7.1, while the extraction and the analysis of the labelled mineral N was performed as described in § 6.3.2 and § 6.3.3.

### 6.4.3 RESULTS AND DISCUSSION

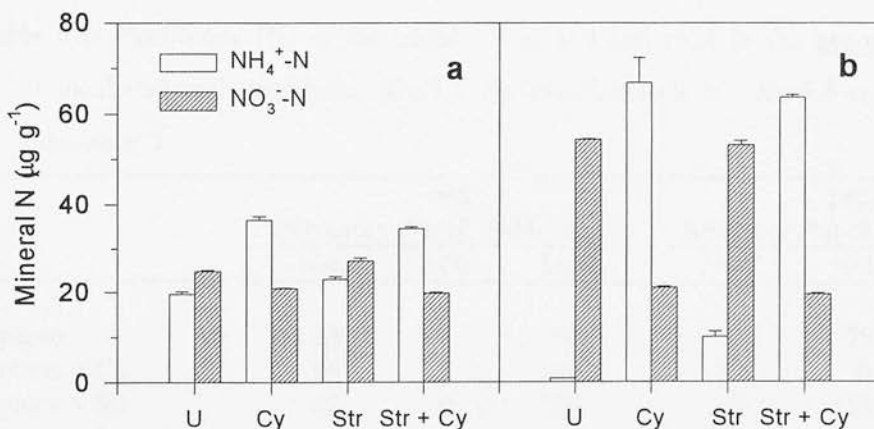
TOTAL MINERAL N: Addition of 7.5 mg of cycloheximide completely blocked any  $\text{NO}_3^-$  production both in the control (Figure 6.3) and in the peptone treated samples (Figure 6.4), while streptomycin did not produce any inhibiting effect on nitrification and acted only as a N substrate, slightly increasing  $\text{NO}_3^-$  production in the samples where it was added. This was more evident in the control samples where no additional source of N other than streptomycin was added. This confirmed previous results (chapter 4 and 5) suggesting that the overall (both autotrophic and heterotrophic) nitrification process is extremely sensitive to high concentrations of cycloheximide but not to high concentrations of streptomycin.

On the other hand, cycloheximide significantly stimulated mineralization, probably with the molecule itself being a substrate for microbial mineralization. However, when cycloheximide was added together with peptone, the  $\text{NH}_4^+$  which accumulated (as nitrification was completely blocked) was less than the sum of  $\text{NH}_4^+$  produced in the samples treated with peptone and cycloheximide alone, suggesting that cycloheximide was acting as a substrate and as an inhibitor (at least partially) of peptone mineralization.

Streptomycin seemed to decrease the  $\text{NH}_4^+$  production deriving from cycloheximide, suggesting that such production could be of bacterial origin. It was not possible from the net rates to define the direct effect of streptomycin on mineralization.



**Figure 6.3** - Total available  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  measured at time zero (a) and after 24 h (b) in the CONTROL samples (without antibiotics-U, with cycloheximide-Cy, with streptomycin-Str and with cycloheximide plus streptomycin-Str + Cy).



**Figure 6.4** - Total available  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  measured at time zero (a) and after 24 h (b) in the PEPTONE treated samples (legend as in Figure 6.3).



**<sup>15</sup>N RESULTS:** In Table 6.1 and 6.2, the <sup>15</sup>N recoveries, in the NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> pools, are reported for the control and the peptone treated samples, calculated using the Ndff equation (Barraclough, 1995a).

**Table 6.1-** Recoveries (%) of the added <sup>15</sup>N at t=0 and t=24 in the control soil preincubated with antibiotics (Cx-7.5 mg cycloheximide g<sup>-1</sup>; Str-3.5 mg streptomycin sulphate g<sup>-1</sup>)

	t=0			t=24		
	Recovery (%) of <sup>15</sup> NH <sub>4</sub> <sup>+</sup> as			Recovery (%) of <sup>15</sup> NH <sub>4</sub> <sup>+</sup> as		
	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>	Total	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>	Total
Control	80	10	90	0.2	73	73
Control + Cx	33	1	34	52	0.8	53
Control + Str	77	7.5	84	3	70	73
Control + Cx + Str	43	1	44	45	0.8	46

	t=0		t=24	
	Recovery (%) of <sup>15</sup> NO <sub>3</sub> <sup>-</sup> as		Recovery (%) of <sup>15</sup> NO <sub>3</sub> <sup>-</sup> as	
	NO <sub>3</sub> <sup>-</sup>		NO <sub>3</sub> <sup>-</sup>	
Control	86		82	
Control + Cx	51		48	
Control + Str	83		77	
Control + Cx + Str	55		50	

**Table 6.2-** Recoveries (%) of the added <sup>15</sup>N at t=0 and t=24 in the peptone treated soil preincubated with antibiotics (Cx-7.5 mg cycloheximide g<sup>-1</sup>; Str-3.5 mg streptomycin sulphate g<sup>-1</sup>)

	t=0			t=24		
	Recovery (%) of <sup>15</sup> NH <sub>4</sub> <sup>+</sup> as			Recovery (%) of <sup>15</sup> NH <sub>4</sub> <sup>+</sup> as		
	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>	Total	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>	Total
Peptone	89	1	90	0.5	78	78
Peptone + Cx	44	0	44	39	0.1	39
Peptone + Str	88	0	88	8	58	64
Peptone + Cx + Str	44	0	44	49	0	49

	t=0		t=24	
	Recovery (%) of <sup>15</sup> NO <sub>3</sub> <sup>-</sup> as		Recovery (%) of <sup>15</sup> NO <sub>3</sub> <sup>-</sup> as	
	NO <sub>3</sub> <sup>-</sup>		NO <sub>3</sub> <sup>-</sup>	
Peptone	81		83	
Peptone + Cx	49		55	
Peptone + Str	84		84	
Peptone + Cx + Str	49		47	

Overall the recoveries were satisfactory, with few exceptions. Where cycloheximide was added very low recoveries were measured, both at  $t=0$  and  $t=24$ . It could be possible that part of the added  $^{15}\text{N}$  was immobilized in the biomass, as cycloheximide also represents a source of C for soil microorganisms (1 mg cycloheximide contains about 639.8  $\mu\text{g}$  C). However, as recoveries at  $t=t$  were generally lower than recoveries at  $t=0$ , the gross rates of mineralization could be calculated with no problems. Generally, where labelled ammonium is added, an increase in the recovery of the label in the ammonium pool could indicate remineralization occurring during the experiment, which would lead to an underestimation of gross rates of mineralization. In the case of labelled nitrate there could be an underestimation of nitrification rates, however, removal and reappearance of  $^{15}\text{NO}_3$  during an experiment is less common.

Two samples showed recoveries at  $t=24$  significantly higher than at  $t=0$ . The first case was represented by the control treated with cycloheximide ( $\text{NH}_4^+$  pool) but the calculated ammonium pool at  $t=24$  (Appendix IV, Table IV.1). was slightly lower than the measured value; the second case was represented by the samples treated with peptone and cycloheximide ( $\text{NO}_3^-$  pool) and in this case the calculated and the measured value were in good agreement (Appendix IV, Table IV.4). Consequently in the former case it is possible that gross mineralization was slightly underestimated, while in the latter the underestimation of gross nitrification rate was probably not significant.

In Table 6.3 are reported the rates of gross mineralization and nitrification calculated using Eqs. 6.1 and 6.4. Results are in accord with what was suggested from net rates.

Cycloheximide blocked any nitrate production, while streptomycin slightly increased gross nitrification in the control. Gross mineralization was stimulated by cycloheximide and to a lesser extent by streptomycin, both acting as a substrate for microbial degradation. When added together the result was a partial inhibition of cycloheximide mineralization, again suggesting a possible role of bacteria in

cycloheximide degradation. On the contrary, in the peptone treated samples addition of antibiotics further stimulated the mineralization rates. It might be possible that the population which was able to mineralize the antibiotics molecules was stimulated by the peptone addition.

**Table 6.3** - Gross rates of mineralization and nitrification in soil combined with/without peptone ( $70 \mu\text{g N g}^{-1}$ ), cycloheximide and streptomycin (Cx - $7.5 \text{ mg cycloheximide g}^{-1}$ ; Str - $3.5 \text{ mg streptomycin sulphate g}^{-1}$ ).

	Mineralization rate $\mu\text{g N g}^{-1}\text{h}^{-1}$	Nitrification rate $\mu\text{g N g}^{-1}\text{h}^{-1}$
Control	$0.36 (\pm 0.02)$	$0.30 (\pm 0.02)$
Control + Cx	$0.80 (\pm 0.04)$	$0.01 (\pm 0.03)$
Control + Str	$0.50 (\pm 0.02)$	$0.36 (\pm 0.02)$
Control + Cx+ Str	$0.57 (\pm 0.05)$	$0.07 (\pm 0.05)$
Peptone	$0.49 (\pm 0.06)$	1.20 --
Peptone + Cx	$1.05 (\pm 0.03)$	$-0.08 (\pm 0.06)$
Peptone + Str	$0.79 (\pm 0.01)$	$1.13 (\pm 0.06)$
Peptone + Cx + Str	$1.01 (\pm 0.04)$	$0.02 (\pm 0.04)$

The size and the  $^{15}\text{N}$  atom % of the  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  pools at  $t=0$ , the mineralization and nitrification rates and the rate at which the ammonium pool changes size (Equ. 6.3), were used in a first simulation model (§ 6.2) to verify if any heterotrophic nitrification might have occurred. The peptone treated samples where nitrification occurred (where no cycloheximide was added) showed a simulated final value of  $^{15}\text{NO}_3^-$  which was higher than the observed, indicating that some nitrate was being produced from an unlabelled source (heterotrophic nitrification). For these samples a second simulation model (§ 6.2) was run to calculate the exact autotrophic and heterotrophic nitrification rates that resulted in a simulated  $^{15}\text{N}$  enrichment in the nitrate pool equal to the measured one.

For the control treatments where  $\text{NO}_3^-$  production occurred (C and C+Str), the simulation gave values of  $^{15}\text{NO}_3^-$  at  $t=24$  lower than those observed (1.51 and 1.17 at.

% simulated, respectively, against 1.91 and 1.68 atomic % measured). Mass balance (Appendix IV, Table IV.2) indicates almost no  $\text{NO}_3^-$  consumption during the 24 h incubation so that a failure in the simulation model due to nitrate loss can be excluded. This failure in the application of the simulation model created problems in interpreting the data, so that it was not possible to evaluate autotrophic and heterotrophic nitrification rates for the control samples.

For the peptone treated samples results are reported in Table 6.4.

**Table 6.4** - Autotrophic and heterotrophic nitrification rates calculated for the peptone samples.

Treatments	Autotrophic nitrification ( $\mu\text{g N g}^{-1} \text{ h}^{-1}$ )	Heterotrophic nitrification ( $\mu\text{g N g}^{-1} \text{ h}^{-1}$ )	Heterotrophic nitrification %
Peptone ( $70 \mu\text{g N g}^{-1}$ )	1.12	0.25	18
Peptone + Cx	0	0	-
Peptone + Str	1.05	0.14	12
Peptone + Cx + Str	0	0	-

The results showed that heterotrophic nitrification accounted for about 18% of the total nitrification at the added peptone concentration. No nitrification was measured in presence of cycloheximide. Streptomycin reduced autotrophic and heterotrophic nitrification rates of a 6% and 44%, respectively, suggesting that the slight decrease of nitrification was mainly due to the bacterial heterotrophic component of the soil microflora.

## 6.5 EFFECTS OF INCREASING CONCENTRATIONS OF CYCLOHEXIMIDE ON THE GROSS RATES OF MINERALIZATION AND NITRIFICATION

### 6.5.1 INTRODUCTION

In this second experiment, increasing concentrations of cycloheximide ( $0.5\text{--}2.5\text{ mg g}^{-1}$  dry soil) were added to the soil, unamended or amended with peptone, and the effects on the gross rates of mineralization and on the autotrophic and heterotrophic component of soil nitrifiers were evaluated by using  $^{15}\text{N}$  isotopic techniques. The experiment also aimed to find some evidence that cycloheximide may act as a simple inhibitor of protein synthesis at low concentrations (main target fungal microflora) and as a toxic molecule at higher concentrations, which could affect also the bacterial soil microbial component, with mechanisms different from the blocking of protein synthesis.

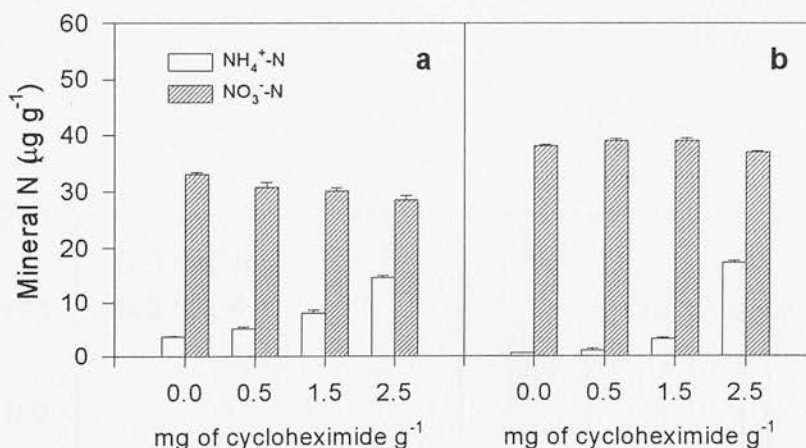
### 6.5.2 EXPERIMENTAL DESIGN

Soil was treated with 0, 0.5, 1.5 and  $2.5\text{ mg g}^{-1}$  of cycloheximide and preincubated as in experiment 1. After 12 h, half of the samples received  $280\text{ }\mu\text{g}$  of peptone-N  $\text{g}^{-1}$ . Immediately after peptone addition, the samples were treated with  $^{15}\text{N}$ . The same treatment as described above was applied to two sets of samples, of which one was incubated at  $25^{\circ}\text{C}$  for 26 h, while the other was immediately extracted after the preparation to measure the mineral N and the isotopic ratio in the samples at *time zero*. After 26 h the second set of samples was extracted as well.

Analyses of mineral N in the soil extract were performed as described in § 2.7.1, while the extraction and the analysis of the labelled mineral N was performed as described in § 6.3.2 and § 6.3.3.

***TOTAL MINERAL N:*** Values of total  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N measured at time zero and after 26 h incubation in the control are presented Figure 6.5.

$\text{NH}_4^+$ -N concentration increased significantly with increasing concentration of cycloheximide. At 0, 0.5 and 1.5 mg of cycloheximide  $\text{g}^{-1}$ , the  $\text{NH}_4^+$ -N which had been mineralized during 12h of preincubation decreased after 26 h incubation. Whereas, at 2.5 mg of cycloheximide it slightly increased. As  $\text{NO}_3^-$  production in the samples treated with cycloheximide was not significantly different from the samples treated with zero cycloheximide, the difference in  $\text{NH}_4^+$  consumption and accumulation might be due to differences in the immobilization rates rather than in the nitrification.



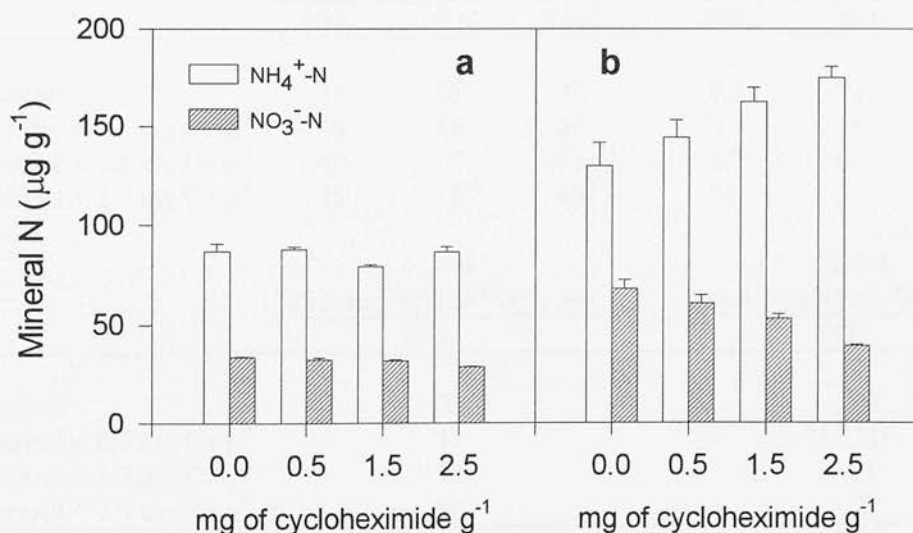
**Figure 6.5** - Total  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N measured in the control soil treated with increasing concentrations of cycloheximide, (a) at time zero and (b) after 26 h incubation.

When peptone was added the concentration of  $\text{NH}_4^+$ -N immediately reached  $86 \mu\text{g N g}^{-1}$  at time zero (Figure 6.6).



Between the moment of peptone addition and the moment of extraction there was a time lag of about 2 hours, necessary for the preparation of the samples with  $^{15}\text{N}$ , which appears to have been sufficient to have a high activity of peptone mineralization, or at least deamination, which, moreover, was not influenced by cycloheximide addition (Figure 6.6 *a*).

An equal quick response for  $\text{NO}_3^-$  production was not observed; the  $\text{NO}_3^-$  concentrations in the peptone-treated and in the control samples at time zero were not significantly different (Figure 6.6 and 6.5). After 26 h, a significant increase in the concentration of  $\text{NO}_3^-$  was observed in both the control and the peptone treated samples. However, while in the former the  $\text{NO}_3^-$  production was not inhibited by cycloheximide addition, in the latter the influence of cycloheximide was evident. Already at  $0.5 \text{ mg g}^{-1}$  of cycloheximide the peptone-induced  $\text{NO}_3^-$  production was reduced by about  $10 \text{ } \mu\text{g N g}^{-1}$ , while at  $2.5 \text{ mg}$  of cycloheximide it was not significantly different from the concentration in the control at  $t=26$  (Figure 6.6*b* and 6.5*b*).



**Figure 6.6** - Total  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  measured in the peptone treated soil with increasing concentrations of cycloheximide, (a) at time zero and (b) after 26 h incubation.

These results suggest, in accord with what observed in previous experiments, that the process of  $\text{NO}_3^-$  production in the control and in the peptone-treated samples were differently influenced by the cycloheximide, the latter being much more sensitive to the antibiotic than the former.

**$^{15}\text{N}$  RESULTS:** The  $^{15}\text{N}$  recoveries in the  $\text{NH}_4^+$  and  $\text{NO}_3^-$  pools, for the control and the peptone treated samples, are given in Table 6.5 and 6.6.

The recoveries in the “control”  $\text{NH}_4^+$  and  $\text{NO}_3^-$  pools were difficult to interpret. Recoveries of  $^{15}\text{NH}_4^+$  decreased from  $t=0$  to  $t=t$ , so that it was possible to calculate gross mineralization rates. However, the overall recovery of  $^{15}\text{N}$  (in the samples treated with  $(^{15}\text{NH}_4)_2\text{SO}_4$ ) increased over time. An hypothesis for what observed could be that the  $^{15}\text{N}$  which was not recovered at  $t=0$  but which reappeared at  $t=26$  was not extractable at  $t=0$  because was blocked in the microbial cells, possibly in the cytoplasm of nitrifiers, being processed.

**Table 6.5-** Recoveries (%) of the added  $^{15}\text{N}$  at  $t=0$  and  $t=24$  in the control soil preincubated with different concentrations of cycloheximide (Cx).

	<b>t=0</b>			<b>t=24</b>		
	Recovery (%) of $^{15}\text{NH}_4^+$ as			Recovery (%) of $^{15}\text{NH}_4^+$ as		
	$\text{NH}_4^+$	$\text{NO}_3^-$	Total	$\text{NH}_4^+$	$\text{NO}_3^-$	Total
Control	39	37	76	0.2	71	71
Control + 0.5 mg Cx $\text{g}^{-1}$	40	18	58	0	68	68
Control + 1.5 mg Cx $\text{g}^{-1}$	40	2	42	0.7	62	63
Control + 2.5 mg Cx $\text{g}^{-1}$	46	0	46	21	32	53

	<b>t=0</b>		<b>t=24</b>	
	Recovery (%) of $^{15}\text{NO}_3^-$ as		Recovery (%) of $^{15}\text{NO}_3^-$ as	
	$\text{NO}_3^-$		$\text{NO}_3^-$	
Control	75		77	
Control + 0.5 mg Cx $\text{g}^{-1}$	71		72	
Control + 1.5 mg Cx $\text{g}^{-1}$	71		71	
Control + 2.5 mg Cx $\text{g}^{-1}$	68		73	

**Table 6.6-** Recoveries (%) of the added  $^{15}\text{N}$  at  $t=0$  and  $t=24$  in the peptone treated soil preincubated with different concentrations of cycloheximide (Cx).

	<b>t=0</b>			<b>t=24</b>		
	Recovery (%) of $^{15}\text{NH}_4^+$ as			Recovery (%) of $^{15}\text{NH}_4^+$ as		
	$\text{NH}_4^+$	$\text{NO}_3^-$	Total	$\text{NH}_4^+$	$\text{NO}_3^-$	Total
Peptone	108	3	111	50	16	66
Peptone + 0.5 mg Cx $\text{g}^{-1}$	112	2	114	58	20	78
Peptone + 1.5 mg Cx $\text{g}^{-1}$	105	1	106	69	11	80
Peptone + 2.5 mg Cx $\text{g}^{-1}$	100	1	101	76	4	80

	<b>t=0</b>		<b>t=24</b>	
	Recovery (%) of $^{15}\text{NO}_3^-$ as		Recovery (%) of $^{15}\text{NO}_3^-$ as	
	$\text{NO}_3^-$		$\text{NO}_3^-$	
Peptone	77		63	
Peptone + 0.5 mg Cx $\text{g}^{-1}$	64		91	
Peptone + 1.5 mg Cx $\text{g}^{-1}$	69		89	
Peptone + 2.5 mg Cx $\text{g}^{-1}$	66		84	

This, however, would affect the simulations used to calculate nitrification, and the effect would be an underprediction of the  $^{15}\text{N}$  in the nitrate pool at time  $t=t$ , because the simulation had started with low initial values of  $^{15}\text{N}$  (see below). The mass balance for the control (Appendix IV, Table IV 5, 6) showed good agreement between calculated and measured values, suggesting that the odd values were not the results of big mistakes in the experimental procedures.

Peptone treated samples showed no problems with recoveries when treated with  $^{15}\text{NH}_4^+$ . On the contrary, the samples treated with  $^{15}\text{NO}_3^-$  showed recoveries of  $^{15}\text{NO}_3^-$  at  $t=t$  higher than  $t=0$ , thus creating problems in calculating gross nitrification rates with Equ. 6.4. As all the data suggested that the  $\text{NO}_3^-$  lost was minimal it was possible to assume that total gross nitrification rates were almost similar to net ones. Net rates were used then in the simulation models to calculate heterotrophic and autotrophic nitrification for peptone samples.

In Table 6.7 are reported gross mineralization and nitrification rates. Increasing concentrations of cycloheximide induced an increase in the mineralization rates, suggesting that cycloheximide, as already observed in previous experiments, is

potentially a substrate for microbial decomposition. Peptone significantly stimulated mineralization and cycloheximide did not seem to interfere significantly with peptone mineralization. The increase in the mineralization rates, when both peptone and cycloheximide were added, seemed to be due to increasing cycloheximide mineralization, while peptone mineralization was stable around  $3.7 \mu\text{g g}^{-1} \text{h}^{-1}$ . This suggests that peptone mineralization, not being affected by cycloheximide, was due to pre-existent enzymatic activity, possibly deaminating extracellular enzymes, or partially to bacterial heterotrophic activity not inhibited by the cycloheximide. This would be in accord with previous results (§ 5.2, § 5.3, § 6.4).

In the control, nitrification was slightly stimulated for concentration of cycloheximide between 0.5 and 1.5  $\text{mg g}^{-1}$ . At 2.5  $\text{mg}$  of cycloheximide it was possible to observe a slight decrease in the nitrification rates.

**Table 6.7** - Gross rates of mineralization and nitrification in soil treated with/without peptone ( $280 \mu\text{g N g}^{-1}$ ) and increasing concentrations of cycloheximide. (Cx - cycloheximide).

	Mineralization rate $\mu\text{g N g}^{-1} \text{h}^{-1}$	Nitrification rate $\mu\text{g N g}^{-1} \text{h}^{-1}$
Control	0.13** ( $\pm 0.01$ )	0.25 ( $\pm 0.02$ )
Control + 0.5 $\text{mg Cx g}^{-1}$	0.39 ( $\pm 0.08$ )	0.31 ( $\pm 0.05$ )
Control + 1.5 $\text{mg Cx g}^{-1}$	0.66 ( $\pm 0.03$ )	0.35 ( $\pm 0.11$ )
Control + 2.5 $\text{mg Cx g}^{-1}$	0.58 ( $\pm 0.01$ )	0.28 ( $\pm 0.03$ )
Peptone	3.70 ( $\pm 0.18$ )	1.51 ( $\pm 0.45$ )*
Peptone + 0.5 $\text{mg Cx g}^{-1}$	4.16 ( $\pm 0.13$ )	1.35 ( $\pm 0.19$ )*
Peptone + 1.5 $\text{mg Cx g}^{-1}$	4.33 ( $\pm 0.10$ )	0.82 ( $\pm 0.25$ )*
Peptone + 2.5 $\text{mg Cx g}^{-1}$	4.55 ( $\pm 0.24$ )	0.43 ( $\pm 0.04$ )*

\* Net rates (see text); \*\* value is too low as  $^{15}\text{N}$  data are not reliable for this measure.

Peptone stimulated nitrification and the addition of increasing concentrations of cycloheximide increasingly reduced nitrification rates. At 2.5  $\text{mg}$  of cycloheximide nitrification rates were comparable to those in the control.

As described in § 6.2 (simulation models) and in § 6.4 (Results section) data were used in a first simulation model to evidence the occurrence of any heterotrophic nitrification.

When the first model was run for the control values, simulated  $^{15}\text{NO}_3$  was always lower than observed  $^{15}\text{NO}_3$ , as already observed in experiment § 5.3. Consequently for the control it was not possible to run the second simulation model to hunt for heterotrophic and autotrophic nitrification. Moreover, given these results, also the first model failed to provide a correct answer. If the explanation given to justify the odd recoveries for the control (page 164-166) is accepted and the model is rerun assuming that all the unrecovered  $^{15}\text{NO}_3$  at  $t=0$  was in the nitrate pool at  $t=0$ , the simulated values came very close to the observed, suggesting that in the control all the nitrification was autotrophic.

As with the first simulation model, all the peptone treatments showed higher simulated than measured values of  $^{15}\text{NO}_3^-$ , so that a second simulation model was run to hunt for heterotrophic and autotrophic nitrification values that resulted in a simulated  $^{15}\text{N}$  enrichment in the nitrate pool equal to the measured one. Results are reported in Table 6.8.

**Table 6.8** - Gross autotrophic and heterotrophic nitrification rates in the peptone treated samples treated with increasing cycloheximide.

Treatments	Autotrophic nitrification ( $\mu\text{g N g}^{-1} \text{ h}^{-1}$ )	Heterotrophic nitrification ( $\mu\text{g N g}^{-1} \text{ h}^{-1}$ )	Heterotrophic nitrification %
Peptone ( $280 \mu\text{g N g}^{-1}$ )	0.64	0.87	56
Peptone + 0.5 Cx	0.85	0.50	37
Peptone + 1.5 Cx	0.50	0.32	38
Peptone + 2.5 Cx	0.41	0.02	4

Heterotrophic nitrification was more than 50% of the overall nitrification induced by peptone addition. What is evident is that cycloheximide differently affected autotrophic and heterotrophic nitrification. The latter started to decrease significantly already at 0.5 mg of cycloheximide and at 2.5 mg it was completely blocked. In contrast, autotrophic nitrification was stimulated by 0.5 mg of cycloheximide, it was slightly decreased at 1.5 mg g<sup>-1</sup>, and at 2.5 mg it was still more than 50% of the nitrification rate in the absence of cycloheximide.

## 6.6 CONCLUSIONS

No experiments have been reported in literature where <sup>15</sup>N techniques are used to evaluate the effects of antibiotic on soil microbial activity and in particular to distinguish between nitrification routes in the presence of antibiotics. Consequently, the possible interference of the two techniques in the soil has not been studied. Despite this, most of the presented results seemed to fit well the proposed analytical equations.

Results showed that the woodland soil had a quite developed population of heterotrophic nitrifiers and that the percentage of heterotrophic nitrification increased with increasing concentrations of peptone. The calculated heterotrophic nitrification was 18% and 56% of total nitrification for 70 and 280 µg peptone-N g<sup>-1</sup>, respectively. Also, the quantity of peptone-N which was mineralized increased with increasing peptone concentration (17% and 32% respectively), probably due to an increased stimulation of the overall microbial activity.

Cycloheximide at low concentrations (0 - 2.5 mg g<sup>-1</sup>) did not seem to have drastic biocidal effects on soil microflora. In the control, it acted both as a substrate and as an inhibitor. It stimulated mineralization and nitrification, and, at the same time, it slightly inhibited nitrification.



Two different biochemical pathways or N routes contributed to peptone-induced nitrate production: the autotrophic and the heterotrophic pathway. These were differently affected by cycloheximide addition. At 2.5 mg g<sup>-1</sup> cycloheximide, heterotrophic nitrification was completely blocked while autotrophic nitrification was still significant. If cycloheximide was sufficiently toxic to prevent any kind of nitrification at 2.5 mg g<sup>-1</sup>, we would have not observed an active autotrophic nitrifiers microflora, especially because autotrophic nitrifiers are generally one of the most sensitive components of soil microflora. This confirms that the drastic N<sub>2</sub>O flux reduction observed at low concentrations of cycloheximide (§ 5.1) is not due to some drastic toxic effect of cycloheximide on autotrophic nitrifying bacteria.

It is more difficult to evaluate if the decrease in the autotrophic nitrifying activity was due to an increasing biocidal effect of cycloheximide on autotrophic nitrifying bacteria or was a real blocking effect on protein synthesis (i.e. blocking of nitrification activity) of the fungal component of the microflora. This would imply the existence of an autotrophic nitrification pathway in fungi, as already discussed in § 5.1.

At higher concentrations cycloheximide became clearly toxic also to the bacterial component of the soil nitrifiers, as no nitrification was measured at 7.5 mg of cycloheximide.

Further support to the hypothesis of an autotrophic pathway of nitrification in fungi could be the fact that 3.5 mg streptomycin only partially reduced both autotrophic and heterotrophic nitrification rates, suggesting that the remaining activity could be due to fungi not inhibited by streptomycin. An alternative explanation for this result could be that the bacterial component was streptomycin resistant but sensitive to cycloheximide. However, in a plate counting experiment carried out on the woodland soil extracts (reported in Appendix V), not a single colony of bacteria was able to grow on plates treated with streptomycin, while comparable concentrations of cycloheximide did not affect bacterial growth. This was evidence against the theory of a major sensitivity of bacteria to cycloheximide rather than streptomycin.

## CYCLOHEXIMIDE INHIBITION ON PEPTONE-INDUCED $\text{N}_2\text{O}$ AND $\text{NO}_3^-$ PRODUCTION ACROSS A SOIL MOISTURE GRADIENT

### 7.1 INTRODUCTION

Soil moisture is one of the main controllers of  $\text{N}_2\text{O}$  emission rates. On the one hand, the soil water content regulates the diffusion of the substrates and consequently the possibility for the microorganisms to interact with them; on the other hand, it regulates  $\text{O}_2$  diffusion through the soil pores and  $\text{O}_2$  supply to the organisms (Skopp *et al.*, 1990) (§ 1.5.2).

The net  $\text{N}_2\text{O}$  emissions from the soil depend on the rate of production, the rate of diffusion out of the soil and the rate of consumption during denitrification (Webster and Hopkins, 1996), all parameters which are influenced by the water content of the soil.  $\text{N}_2\text{O}$  production itself has been found to have a very different dependence on the water content of the soil when it derives from nitrification than from denitrification (Linn and Doran, 1984).

When antibiotics are used in soil to estimate fungal versus bacterial activity, the soil moisture is usually held at 55-60% of WHC or at 60% of WFPS (Anderson and

Domsch, 1973; Stamatiadis *et al.*, 1990; Wardle and Parkinson, 1990; Landi *et al.*, 1993; Badalucco *et al.*, 1994), which are considered to be optimal values for aerobic processes in soil (Linn and Doran, 1984). However, to test the potential of soil for heterotrophic nitrification, soil slurries have also been used, to which peptone was added as an N source (Schimel *et al.*, 1984; Landi *et al.*, 1992).

There is not a definitive conclusion on the pathway of nitrification led by heterotrophic microorganisms. Consequently, it is also not clear what is the dependence of the process by soil O<sub>2</sub> content, or which is the contribution of different microbial groups, such as fungi or different kinds of heterotrophic bacteria at different O<sub>2</sub> partial pressures, or how the water content could influence each of these groups activity and the overall heterotrophic nitrification process.

It could be hypothesised that organisms or processes which are more sensitive to decreasing concentrations of oxygen could be less favoured in incubation conditions which reduce O<sub>2</sub> diffusion. Soil fungi for example are mainly aerobic microorganisms (Cochrane, 1958, Curtis, 1969). Bollag and Tung (1972) found that fungi were growing well on various media in a well aerated atmosphere, but when oxygen was limiting, the yield of the cells was ten times lower, and even lower in anaerobic conditions. Consequently, it could be possible that the fungal:bacterial nitrification activity ratio could decrease for increasing water contents. Consequently, the water content at which the soil is incubated could be very important if we want to determine the fungal contribution to nitrification and N<sub>2</sub>O emissions. High fluxes, not inhibited by cycloheximide, in a waterlogged soil could lead to the general conclusion that fungi are not contributing to the emissions in that particular soil, while the same soil could show a different response to cycloheximide at lower water contents.

The following experiment was intended to test the effectiveness of cycloheximide inhibition on N<sub>2</sub>O fluxes and NO<sub>3</sub><sup>-</sup> peptone-induced production, across a soil moisture gradient.

Water filled pore space (WFPS) was used as the parameter to express the quantity of water present in the soil, as it directly relates to diffusivity and also accounts for variation in total porosity among soils (§ 2.7.6). As gaseous diffusion is one of the main controlling factors on the processes which lead to N<sub>2</sub>O emissions, WFPS appeared to be more appropriate than  $\theta_g$  and  $\theta_v$  to express the soil water content.

## 7.2 EXPERIMENTAL DESIGN

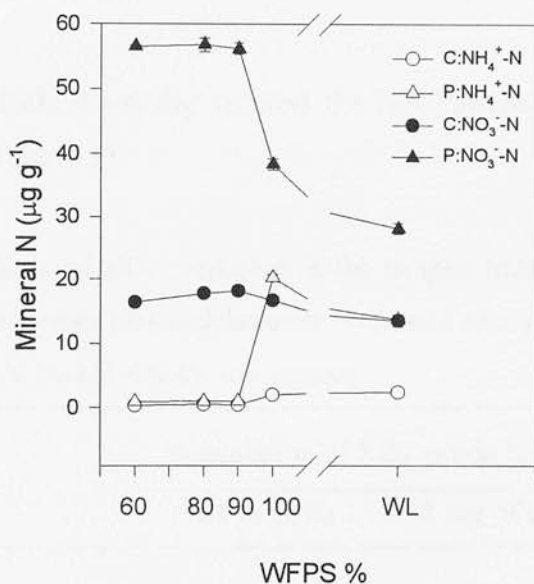
The woodland soil was sampled from the top 10 cm, sieved (2 mm) and pre-incubated (12 h) with 2 mg of cycloheximide g<sup>-1</sup> (§ 4.2.2). The next morning peptone (70 µg of N g<sup>-1</sup>) was added to half of the samples. Soil samples of 20 g each were incubated in 1 l air-tight jars. Deionized water was added to bring the samples to 60%, 80%, 90% and 100% of the water filled pore space (WFPS). For each water content, three replicates were incubated with zero C<sub>2</sub>H<sub>2</sub>, three with 0.1% C<sub>2</sub>H<sub>2</sub> and three with 10% C<sub>2</sub>H<sub>2</sub> (Davidson *et al.*, 1986) to distinguish respectively total N<sub>2</sub>O production, autotrophic versus non-autotrophic N<sub>2</sub>O production, and N<sub>2</sub> production. At the same time, soil was also waterlogged and incubated in the form of a soil slurry. 10 g of soil, previously pre-incubated with cycloheximide and then amended with peptone, as described for the other samples, were slurried in 50 ml of deionized water. The ratio 1:5 soil:solution has been used to test nitrification in soil slurries (Schimel *et al.*, 1989); however, the solution was usually a buffer solution also containing the N substrate. As it was intended to test the influence of increasing quantities of water on the measurements of fungal activity without changing any other condition, the soil was slurried using simple deionized water. As for the other samples, three replicates were incubated with zero C<sub>2</sub>H<sub>2</sub>, three with 0.1% C<sub>2</sub>H<sub>2</sub> and three with 10% C<sub>2</sub>H<sub>2</sub>.

All samples were incubated at 25°C for 24 h, after which gas samples were taken with greased glass syringes and immediately analysed for N<sub>2</sub>O by ECD gas chromatography. The soil was then extracted for the analysis of mineral N. In the extraction procedure, 20 g of soil are usually shaken with 100 ml of 1 M KCl. To extract mineral N from the soil slurries with a KCl solution having the same molarity

as for the other samples, 50 ml of 2 M KCl were added to the slurry (final concentration was then 1 M KCl). For the routine analysis see chapter 2.

### 7.3 RESULTS AND DISCUSSION

In Figure 7.1 the values of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  measured in the control and in the peptone treated samples after 24 h incubation are reported.



**Figure 7.1** -  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  extracted from the control (C) and the peptone treated (P) samples after 24 h. (WL refers to 10 g soil 50 ml<sup>-1</sup> water).

$\text{NO}_3^-$  concentration was significantly higher in the peptone treated samples than in the control, throughout all the moisture gradient. However while  $\text{NO}_3^-$  concentration at 60%, 80% and 90% WFPS was not significantly different, at 100% WFPS it significantly decreased, and such a decrease was even more pronounced when the soil was waterlogged (WL). In the soil slurry (WL), the  $\text{NO}_3^-$  concentration was about 40% of the  $\text{NO}_3^-$  concentration at 60-90% WFPS. The same drastic reduction was not observed in the control, where however the  $\text{NO}_3^-$  concentration in 24h was always quite low (6-8  $\mu\text{g N g}^{-1}$ ).

$\text{NH}_4^+\text{-N}$  concentration in the peptone treated soil was very low between 60% and 90% of WFPS, it increased at 100% WFPS and decreased again when the soil was waterlogged. The increase at 100% WFPS could have been mainly due to the reduced nitrification rate of the soil; indeed, the increase in  $\text{NH}_4^+\text{-N}$  was comparable to the decrease of  $\text{NO}_3^-\text{-N}$  ( about  $20 \mu\text{g N g}^{-1}$ ). The reduction observed when soil was water logged could be due to a lower rate of mineralization of the added peptone-N (as otherwise we should have observed a further increase of  $\text{NH}_4^+$  in soil as compared with 100% WFPS treatments).

Addition of 0.1%  $\text{C}_2\text{H}_2$  drastically reduced the  $\text{NO}_3^-$  production across the whole moisture gradient (Table 7.1).

**Table 7.1** - Inhibition of  $\text{NO}_3^-$  production in the samples treated with peptone plus 0.1%  $\text{C}_2\text{H}_2$  or peptone plus cycloheximide, expressed as a percentage of the  $\text{NO}_3^-$  found in the soil treated with the sole peptone.

	% inhibition of $\text{NO}_3^-$ production in presence of	
	0.1% $\text{C}_2\text{H}_2$	2 mg of cycloheximide $\text{g}^{-1}$
60% WFPS	92.9	26.4
80% WFPS	94.8	16.9
90% WFPS	94.4	10.6
100% WFPS	99.0	9.4
Waterlogged (WL)	97.0	4.6

Reduction was in the range of 92-99% and was almost equally effective at any water content, suggesting that diffusion of 0.1%  $\text{C}_2\text{H}_2$  in soil was not limited by the increasing soil moisture. 10%  $\text{C}_2\text{H}_2$  did not have a significantly different effect on  $\text{NO}_3^-$  production as compared with 0.1%  $\text{C}_2\text{H}_2$  (data not shown).



The results suggest that most of the  $\text{NO}_3^-$  was produced throughout the moisture gradient by a process involving ammonia-monooxygenase.

The inhibition percentage of  $\text{NO}_3^-$  production, induced by the addition of 2 mg of cycloheximide  $\text{g}^{-1}$ , decreased drastically for increasing moisture contents of the soil. Already at 80% of WFPS the effectiveness of cycloheximide inhibition was reduced by about one third. In the soil slurry cycloheximide inhibition was only 18% of the inhibition at 60% of WFPS.

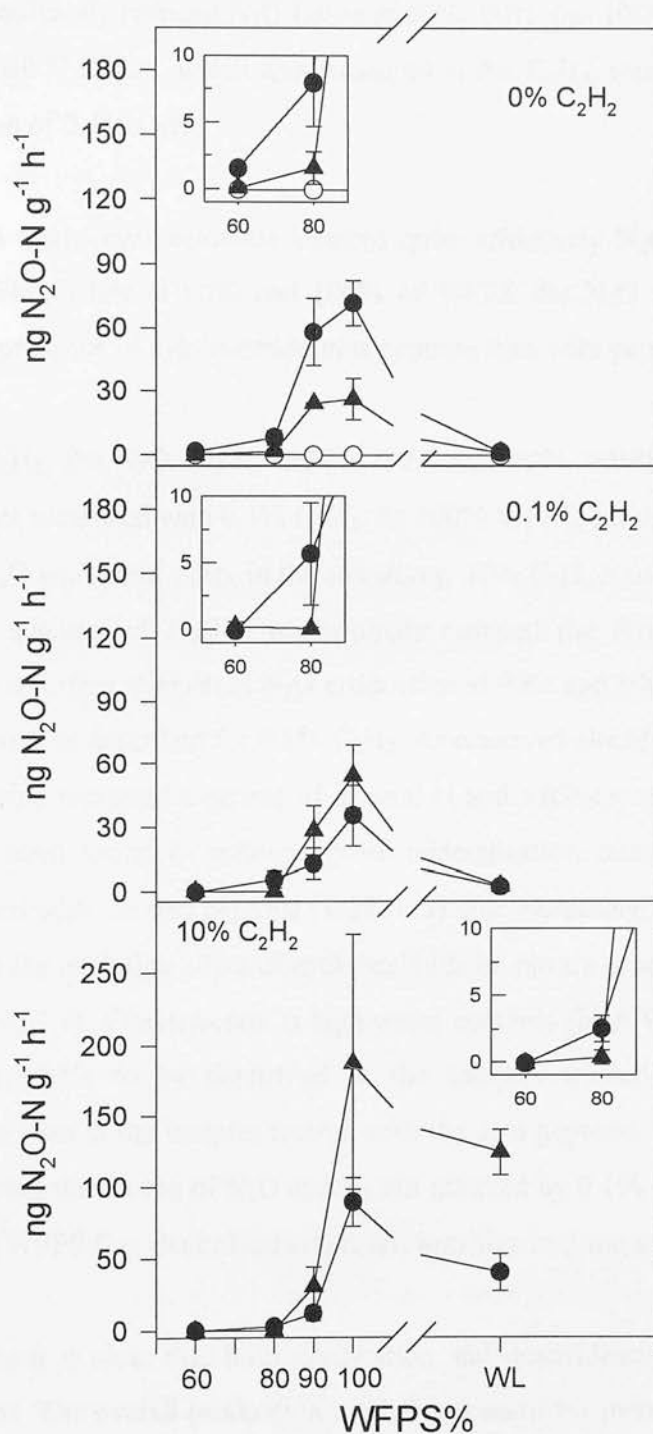
Peptone addition to the soil induced a significant increase in  $\text{N}_2\text{O}$  emission rates (Figure 7.2) as compared with the control. At 0%  $\text{C}_2\text{H}_2$ , the peptone-induced  $\text{N}_2\text{O}$  production increased exponentially up to 90% WFPS; between 90% and 100% WFPS it increased further but at a slower rate, reaching  $71.9 \text{ ng N}_2\text{O-N g}^{-1} \text{ h}^{-1}$ , and finally it declined almost to zero in the soil slurry.

Cycloheximide was very effective in reducing the  $\text{N}_2\text{O}$  flux at 60% and 80% WFPS (see the inset in Figure 7.2 at 0%  $\text{C}_2\text{H}_2$ ). However, at 90% and 100% WFPS the effect of cycloheximide inhibition on the  $\text{N}_2\text{O}$  flux decreased.

In the control, a negative  $\text{N}_2\text{O}$  flux was measured, which indicated  $\text{N}_2\text{O}$  consumption. Such a consumption increased with increasing water content of the soil (Table 7.2).

**Table 7.2** -  $\text{N}_2\text{O}$  fluxes measured from the control (untreated) woodland soil across a soil moisture gradient. (One standard error in brackets).

Water filled pore space - WFPS	$\text{N}_2\text{O}$ flux ( $\text{ng N g}^{-1} \text{ h}^{-1}$ )
60%	- 0.109 ( $\pm 0.013$ )
80%	- 0.113 ( $\pm 0.060$ )
90%	- 0.126 ( $\pm 0.021$ )
100%	- 0.156 ( $\pm 0.011$ )
slurry (10g soil 50 $\text{ml}^{-1}$ water)	- 0.304 ( $\pm 0.030$ )



**Figure 7.2** -  $\text{N}_2\text{O}$  emission rates from the control (○), the peptone treated (●) and the peptone plus cycloheximide (▲) treated samples, in presence of 0%, 0.1% or 10%  $\text{C}_2\text{H}_2$ .

When 0.1% C<sub>2</sub>H<sub>2</sub> was added to the peptone treated samples, it blocked completely the N<sub>2</sub>O flux at 60% WFPS, which decreased from 1.50 ng N g<sup>-1</sup> h<sup>-1</sup> to -0.13 ng N g<sup>-1</sup> h<sup>-1</sup>, and it significantly reduced N<sub>2</sub>O fluxes at 80%, 90% and 100% WFPS. In the soil slurry, the small N<sub>2</sub>O flux, which was measured at 0% C<sub>2</sub>H<sub>2</sub>, was completely blocked by the addition of 0.1% C<sub>2</sub>H<sub>2</sub>.

Also at 0.1% C<sub>2</sub>H<sub>2</sub>, cycloheximide blocked quite effectively N<sub>2</sub>O emissions at 60% and 80% WFPS, while at 90% and 100% of WFPS the N<sub>2</sub>O emission rates were higher in the presence of cycloheximide plus peptone than with peptone alone.

With 10% C<sub>2</sub>H<sub>2</sub>, the N<sub>2</sub>O fluxes at 60%, 80% and 90% WFPS were not different from the fluxes measured with 0.1% C<sub>2</sub>H<sub>2</sub>. At 100% WFPS, however, there was a big increase in N<sub>2</sub>O emissions. Also, in the soil slurry, 10% C<sub>2</sub>H<sub>2</sub> induced the formation of a significant quantity of N<sub>2</sub>O. Cycloheximide reduced the flux at 60% and 80% WFPS, while it further stimulated N<sub>2</sub>O production at 90% and 100% WFPS as well as in the soil slurry, as described for 0.1% C<sub>2</sub>H<sub>2</sub>. As observed already in chapter 4, 5 and 6, cycloheximide represent a source of mineral N and addition of cycloheximide plus peptone has been found to enhance gross mineralization rates as compared with samples treated with the sole peptone (Table 6.3). For increasing WFPS % it has been observed that the inhibiting effect of cycloheximide on nitrate production was strongly reduced (Table 7.1). Consequently at high water contents (high WFPS) there is more mineral N available to be denitrified in the samples treated with peptone and cycloheximide than in the samples treated with the sole peptone. However the results also suggest that the source of N<sub>2</sub>O and N<sub>2</sub> not affected by 0.1% C<sub>2</sub>H<sub>2</sub> and stimulated by increasing WFPS (i.e. denitrification) is not sensitive to 2 mg cycloheximide g<sup>-1</sup>.

From the data it is clear that both nitrification and denitrification were involved in N<sub>2</sub>O emissions. The overall production of N<sub>2</sub>O increased for increasing soil moisture, reaching an optimum at 100% WFPS, but declined significantly in the soil slurry (Figure 7.2, Table 7.3).

**Table 7.3** - Emission rates of total N<sub>2</sub>O, N<sub>2</sub> and (N<sub>2</sub>O+N<sub>2</sub>)-N deriving from denitrification, measured from the woodland soil across a moisture gradient.

WFPS %	Total N <sub>2</sub> O-N (ng N g <sup>-1</sup> h <sup>-1</sup> )	N <sub>2</sub> (ng N g <sup>-1</sup> h <sup>-1</sup> )	*D-(N <sub>2</sub> O+N <sub>2</sub> )-N (ng N g <sup>-1</sup> h <sup>-1</sup> )
60	1.50	0	0
80	7.93	0	3.50
90	58.07	0	12.50
100	71.91	54.02	89.35
WL (10g soil 50 ml <sup>-1</sup> water)	1.16	39.23	40.13

\*D: denitrification

N<sub>2</sub> production was not detected till the soil reached 100% WFPS, and was still produced but at a lower rate in the waterlogged soil, probably because less NO<sub>3</sub><sup>-</sup> was available for denitrification (Table 7.3, Figure 7.1). Denitrification did not produce significant (N<sub>2</sub>O+N<sub>2</sub>)-N at 60% WFPS, while gaseous N increased exponentially up to 100% WFPS and significantly declined in the soil slurry ( Table 7.3).

In Table 7.4 is reported the contribution of autotrophic and non-autotrophic processes to the total N<sub>2</sub>O flux measured.

**Table 7.4** - Peptone-induced N<sub>2</sub>O fluxes deriving from autotrophic or non-autotrophic processes, or measured from the soil treated with cycloheximide. Calculations are made on averages.

	Autotrophic	Non-autotrophic	+ cyclohex.
WFPS	N <sub>2</sub> O (ng N g <sup>-1</sup> h <sup>-1</sup> )	N <sub>2</sub> O (ng N g <sup>-1</sup> h <sup>-1</sup> )	N <sub>2</sub> O (ng N g <sup>-1</sup> h <sup>-1</sup> )
60	1.63	0	0.05
80	4.43	3.50	1.54
90	45.12	12.50	24.03
100	36.58	35.33	26.09
WL (10g soil 50 ml <sup>-1</sup> water)	0.26	0.90	0.99

Both the processes increased with increasing soil water content till 90-100% of WFPS and both declined drastically in the soil slurry. Autotrophic nitrification was the main process producing  $\text{N}_2\text{O}$  at 90% of WFPS; while at 100% WFPS autotrophic and non-autotrophic  $\text{N}_2\text{O}$  emissions were comparable but the total N flux ( $\text{N}_2\text{O}+\text{N}_2$ ) deriving from denitrification was far bigger than the autotrophic emission of  $\text{N}_2\text{O}-\text{N}$  (Table 7.3, 7.4). In the soil slurry the autotrophic contribution to  $\text{N}_2\text{O}$  emissions was almost zero, while the main product of denitrification was  $\text{N}_2$  (Table 7.3, 7.4).

Kralova *et al.* (1992), using soil suspension at controlled redox potentials, found that  $\text{N}_2\text{O}$  was the main gaseous product when the soil was at intermediate redox potentials (+ 200mV) and that such production was much bigger than  $\text{N}_2\text{O}$  production at higher redox potentials (around + 400 mV). Moreover, they found that  $\text{N}_2$  was the main N species evolved only under strongly reduced conditions (-200 mV - 0 mV). In their experiment they considered denitrification as the main source of  $\text{N}_2\text{O}$  and  $\text{N}_2$ .

$\text{N}_2\text{O}$  produced by autotrophic microorganisms had been reported to increase for increasing water contents and decreasing  $\text{O}_2$  contents in the soil (Bremner and Blackmer, 1980; Goreau *et al.*, 1980). In both sediments and cores, the maximum  $\text{N}_2\text{O}$  concentration derived from autotrophic production has been found at depths of most active nitrification (i.e. active nitrite and nitrate formation) and reduced, but not zero, oxygen concentrations (Khdyer and Cho, 1983; Knowles *et al.*, 1981, cited in Poth and Focht, 1985). In these condition  $\text{O}_2$  is still present to produce significant quantities of  $\text{NO}_2^-$ , which is the substrate reduced to  $\text{N}_2\text{O}$ , and hydroxylamine, which is the electron source for  $\text{NO}_2^-$  reduction. At the same time, oxygen tension is low enough to favour the process of  $\text{NO}_2^-$  reduction versus  $\text{O}_2$  reduction. The use of nitrite as a terminal electron acceptor by autotrophic microorganisms has been proposed as a way for “conserving oxygen for the initial mixed-function oxidase step of ammonium oxidation, as a way of removing the toxic product nitrite, as a way to decrease competition for oxygen with nitrite oxidisers” (Poth and Focht, 1985).

The presented data are consistent with what is reported in the literature. It can be assumed indeed, that up to a certain level of WFPS (around 90%) the soil was still sufficiently aerobic to favour autotrophic  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  production, being also further facilitated by a higher substrate solubility. A slight decrease in oxygen tension could have further favoured  $\text{N}_2\text{O}$  production.

$\text{N}_2\text{O}$  deriving from denitrification peaked at 100% but was already detectable at 80% WFPS. Several denitrifier organisms have been reported to be able to co-respire oxygen and  $\text{NO}_3^-$  while producing mainly  $\text{N}_2\text{O}$  (versus  $\text{N}_2$ ) (Robertson and Kuenen, 1990; Thomas *et al.*, 1994). This process can be expected to increase for decreasing oxygen concentration, as appeared to occur here (Table 7.3). Though  $\text{O}_2$  concentration was not measured in the present experiment, the high increase in  $\text{N}_2$  production for increasing WFPS % was an indirect demonstration of a decreasing  $\text{O}_2$  concentration.  $\text{N}_2$  production was significant only around 100% WFPS. Usually it is reported that a significant quantity of  $\text{N}_2$  is produced as the oxygen decreases and as the water content increases, as nitrous oxide reductase activity is blocked by  $\text{O}_2$  and a higher water content allows more  $\text{N}_2\text{O}$  to be trapped and reduced to  $\text{N}_2$ . Webster and Hopkins (1996) found that the rate of  $\text{N}_2\text{O}$  production was higher in a wetter soil than in a drier soil, but because  $\text{N}_2\text{O}$  consumption was also greater in the wetter soil, the net  $\text{N}_2\text{O}$  production was not different in the two soils. Moreover,  $\text{N}_2\text{O}$  is 25 times more soluble in water than  $\text{N}_2$  (Merck Index, 1960) so that the low  $\text{N}_2\text{O}$  fluxes measured from the soil slurries could be either due to a higher degree of  $\text{N}_2\text{O}$  reduction or to a slower diffusion of  $\text{N}_2\text{O}$  to the head space through the water.

The addition of cycloheximide to the soil resulted in a complete inhibition of the autotrophic flux at 60% WFPS (Table 7.4). From 80% WFPS a reduced efficiency of cycloheximide inhibition of the  $\text{N}_2\text{O}$  flux was observed. This could be the result of different processes. On the one hand, for increasing water contents and decreasing  $\text{O}_2$  contents, fungal nitrification could become less important, while bacterial autotrophic nitrification could be favoured instead. As a result, bacterial autotrophic  $\text{N}_2\text{O}$  production could mask the decrease in  $\text{N}_2\text{O}$  fungal production. On the other hand, the



N<sub>2</sub>O produced by denitrification could overlap with N<sub>2</sub>O produced by any eventual autotrophic fungal nitrification, making the results difficult to interpret, a further complication being the fact that cycloheximide itself significantly stimulated the denitrification activity.

## 7.4 CONCLUSIONS

The results confirmed that water content has a big influence on the composition of gaseous N products evolved from the soil and on the relative quantity of N<sub>2</sub>O which is evolved. Different processes were shown to give different contributions to the overall N<sub>2</sub>O flux at increasing water contents.

The NO<sub>3</sub><sup>-</sup> production was not affected by soil moisture up to 90% WFPS, probably because the soil was still sufficiently aerobic. Indeed the soil used is a very light soil, moreover it was sieved, which further reduced soil compaction. However, a partial decrease of O<sub>2</sub> for increasing moisture must have happened, as confirmed by the trends of autotrophic N<sub>2</sub>O production and denitrification. Denitrification became the main process of gaseous N production at WFPS > 100%.

Cycloheximide showed a good efficiency for reducing N<sub>2</sub>O and NO<sub>3</sub><sup>-</sup> production only at 60% of WFPS, while it showed a progressive reduction in inhibitory efficiency with increasing soil moisture. This could be due either to a reduced contribution of fungi versus bacteria to N<sub>2</sub>O and NO<sub>3</sub><sup>-</sup> production for increasing water contents, or anyway to a partial overlapping of N<sub>2</sub>O production by different processes. Consequently care must be taken when cycloheximide is used to test fungal contribution to nitrification and N<sub>2</sub>O emissions, and results obtained in a soil at 60% WFPS could be not easily comparable with results obtained with a test done on soil slurries.

## DISCUSSION AND CONCLUSIONS

### 8.1 FIELD N<sub>2</sub>O FLUXES IN THE WOODLAND AND IN THE ARABLE SOIL

Results from field experiments indicated two different patterns of N<sub>2</sub>O production. Measurements by manual chambers showed very low fluxes (1.4 - 1.55 g N<sub>2</sub>O-N ha<sup>-1</sup> d<sup>-1</sup>) in both fields, throughout the year. Other authors have reported low N<sub>2</sub>O fluxes in light textured soils (sandy soils), from natural or agricultural sites subjected to fertilization, located in the same climatic region (Skiba *et al.*, 1993a; Skiba *et al.*, 1993b; McTaggart *et al.*, 1994), and they also found extremely low emissions, when compared with soils of different textures, even after fertilisation events.

The measured fluxes were not significantly correlated with any environmental parameter. Though other authors have found weak correlation between N<sub>2</sub>O fluxes and environmental parameters (Sheperd *et al.*, 1991; Clayton *et al.*, 1997) the lack of correlation might have been due to the extremely low fluxes measured. However, a lack of correlation does not necessarily mean that the processes which were responsible for this low but constant flux throughout the year were not influenced by environmental conditions. Indeed, fluxes showed a certain seasonal pattern, with two peaks, in spring and in winter, which are typical of trends of microbial activity in general, which is influenced by the overall combination of environmental variables.

Also soil WFPS, temperature,  $\text{NO}_3^-$  concentration, showed a similar seasonal trend with  $\text{N}_2\text{O}$ , despite not being significantly correlated.

Nitrification, either autotrophic (and) or heterotrophic might have been the source of the above mentioned fluxes as nitrification is a process which generally shows a moderate variability, due to the fairly homogeneous distribution of aerobic sites, nitrifiers and substrate, it is generally considered to produced rather low  $\text{N}_2\text{O}$  fluxes (as opposed to denitrification) and moreover in light textured soils it is generally regarded as the most common process of  $\text{N}_2\text{O}$  production (Tortoso and Hutchinson, 1990; Davidson, 1992; Hutchinson 1993).

However, in light textured soil it is also possible that centres of denitrification will form associated with “hot spots” of microbial activity where organic matter is more abundant (Parkin, 1987; Christensen *et al.*, 1990a; Smith, 1990). High flux rates of  $\text{N}_2\text{O}$  can be associated with these events, especially because it is more probable that  $\text{N}_2\text{O}$  diffuses to the atmosphere without being further reduced, as the soil around these small anaerobic aggregates is aerobic (Thomas *et al.*, 1994; Webster and Hopkins, 1996). Indeed, the fertilization experiment in the woodland and the incubation of woodland soil cores showed a source of  $\text{N}_2\text{O}$  which was stimulated by the presence of organic matter and mineral N and which was highly variable. Denitrification or anyway a process stimulated by presence of available C, N and lower oxygen concentration might have been responsible for the big peaks which were measured during the first days after fertilisation in the treated woodland plot by automated chambers. High spatial and temporal variability is generally correlated to  $\text{N}_2\text{O}$  production from denitrification activity, which can exhibit coefficients of variation in the range 100%-500% (Folorunso and Rolston, 1984; Parkin *et al.*, 1987; Christensen *et al.* 1990a,b; Parkin 1990). This might create the problem of covering a soil surface which is large enough to have a representative gas flux. Though in the studied sites, the number of chambers was quite small the total covered area was about 7416 cm<sup>2</sup>. Christensen *et al.* (1990a) carried out a study of spatial variability of denitrification using 30 chambers of 10 cm diameter, with a total area of 2355 cm<sup>2</sup>. In

terms of  $\text{N}_2\text{O}$  emissions,  $7416 \text{ cm}^2$  distributed over 3 chambers should have provided a reasonable indication of the average  $\text{N}_2\text{O}$  flux, although the coefficient of variability could be estimated with less precision than with 30 chambers of smaller area. The fact that the  $\text{N}_2\text{O}$  peaks observed with automated chambers were not observed with manual chambers in the same treated plot, might be related more to a problem of temporal variability (low sampling frequency) rather than spatial variability (not enough chambers). Indeed, temporal variability was quite high: variations in the flux of up to  $40 \text{ g N ha}^{-1} \text{ day}^{-1}$  were recorded over a few hours by automated chambers. The possibility of missing significant peaks due to the high temporal variability of soil fluxes might also explain the low fluxes measured in the field throughout the year by manual chambers in both sites.

## 8.2 MICROBIAL PROCESSES INVOLVED IN $\text{N}_2\text{O}$ EMISSIONS

Soils were tested for potential  $\text{N}_2\text{O}$  production, nitrification and denitrification, where “potential” refers to the rate of processes when soils were incubated under a set of defined conditions after amending with different N substrates (Killham, 1990). Results showed that more than one mechanism was involved in  $\text{N}_2\text{O}$  emissions and that those mechanisms had a different importance in the two soils.

Added  $\text{NH}_4^+$  was quickly consumed in both soils, suggesting an efficient nitrifier population, though only the woodland soil showed a significant net production of  $\text{NO}_3^-$  ( $2.53 \mu\text{g N g}^{-1} \text{ h}^{-1}$  in the woodland against  $0.02 \mu\text{g N g}^{-1} \text{ h}^{-1}$  in the arable land).  $\text{N}_2\text{O}$ -N production was of the same order of magnitude in the two soils (0.25%-0.35% of the added  $\text{NH}_4^+\text{-N}$ ), but was quite low. The arable soil showed much higher  $\text{N}_2\text{O}$  emissions when the soil was provided with  $\text{NO}_2^-$  or  $\text{NO}_3^-$  and a C source, suggesting a very active denitrifier population. In contrast, the woodland soil showed a very low denitrification activity and a much higher  $\text{N}_2\text{O}$  production derived from the oxidation of  $\text{NH}_4^+$  and reduction of  $\text{NO}_2^-$  by some processes probably mediated by autotrophic or heterotrophic nitrifiers or fermentative  $\text{NO}_2^-$  reducers.

In particular the  $\text{N}_2\text{O}$  flux stimulated by  $\text{NO}_2^-$  addition and inhibited by  $\text{C}_2\text{H}_2$  (about  $14 \text{ ng N}_2\text{O-N g}^{-1} \text{ h}^{-1}$ ) was supposed to derive from autotrophic nitrification of the  $\text{NH}_4^+\text{-N}$  accumulated in the soil ( $16.6 \text{ } \mu\text{g NH}_4^+\text{-N g}^{-1}$ ) as a result of  $\text{NO}_2^-$  reduction to  $\text{NH}_4^+$ , as observed in Table 4.1. However, the measured  $\text{N}_2\text{O}$  flux was higher than in the AS treatment, where  $70 \text{ } \mu\text{g NH}_4^+\text{-N g}^{-1}$  were added, suggesting that  $\text{NO}_2^-$  might have stimulated the activity of autotrophic nitrifiers in some way. Poth and Focht (1985) proposed that Nitrosomonas europea produces  $\text{N}_2\text{O}$  by using  $\text{NO}_2^-$  as electron acceptor in microsites where the oxygen concentration is low; however, the electrons are derived from the first step of the nitrifying process, which involves  $\text{NH}_4^+$ ,  $\text{NH}_2\text{OH}$  and ammonia-monooxygenase (Wood, 1986). Blocking the nitrification of ammonia oxidation with inhibitors would eliminate the source of electrons (hydroxylamine) for nitrite reduction and hence  $\text{N}_2\text{O}$  production. Indeed, production of  $\text{N}_2\text{O}$  by such microorganisms has been found to be particularly enhanced by addition of  $\text{NH}_4^+$  and electron donors and to be correlated with  $\text{NH}_4^+$  but not with  $\text{NO}_3^-$  additions (Blackmer et al., 1980; Abielovich and Vonshak, 1992).

The  $\text{N}_2\text{O}$  flux not inhibited by  $\text{C}_2\text{H}_2$  in the soil treated with  $\text{NO}_2^-$  and  $\text{NO}_2^-$  plus glucose was attributed to heterotrophic nitrification rather than to denitrification (§ 4.1). Where no glucose was added the organic matter present in the soil could have provided a potential source of electrons for heterotrophic nitrification (not inhibited by  $\text{C}_2\text{H}_2$ ), whereas when glucose was added together with  $\text{NO}_2^-$  a more readily available source of electrons significantly increased  $\text{N}_2\text{O}$  emissions. Several heterotrophic microorganisms such as Arthrobacter sp. and Alcaligenes faecalis were found to be able to nitrify, but as the process consumed energy it was carried out only when a source of energy was supplied (Castignetti et al., 1990). The heterotrophic bacterium Tiosphera pantotropha was found to nitrify only when acetate was added as electron donor (Robertson et al. 1988).

In the present work the incubating conditions were chosen to represent conditions not too far from what found in the field rather than to measure potential rates of different processes which produce  $\text{N}_2\text{O}$ . Consequently, though denitrifiers can be considered



ubiquitous, their activity might not have prevailed in the experimental and in most of the field conditions.

A well developed population of heterotrophic nitrifiers in the woodland soil was also demonstrated by experiments with  $^{15}\text{N}$  described in chapter 6. The balance between autotrophic and heterotrophic nitrification in the soil was influenced by the concentration of organic N. The heterotrophic activity increased from 18% to 56% of the total nitrification activity when the peptone concentration was increased from 70 to  $280\ \mu\text{g N g}^{-1}$ . These results underline the potential role of heterotrophic nitrification in the woodland soil. Moreover, the use of antibiotics coupled with the  $^{15}\text{N}$  technique showed that fungi (and probably yeasts) were the main component of the heterotrophic nitrifier population which was able to oxidise peptone-N.

The significance of fungal nitrification in forest soils and other soils is at present the subject of much speculation (Killham, 1990). As outlined in the introduction and in chapter 5, there is some evidence for both an inorganic (Aleem, 1975) and an organic pathway of nitrification (Doxtader, 1965; Wood, 1987). Soil fungi have been found to be able to produce significant amounts of  $\text{NO}_3^-$  both from ammonium and peptone in continuous cultures (Remacle, 1977). It has been observed that several ectomycorrhizal-forming basidiomycetes isolated from coniferous soils can nitrify both inorganic and organic forms of nitrogen (Hoyle, pers. comm.). The possibility of significant fungal nitrification in the woodland soil is particularly interesting as heterotrophic bacterial and fungal nitrification has been assumed to be characteristic mainly of acid soils (Bollag and Tung, 1972; Bleakley and Tiedje, 1982; Stroo *et al.*, 1986; Robertson and Tiedje, 1987; Martikainen 1995).

The two different biochemical pathways or N routes which contributed to peptone-induced nitrate production, i.e. the autotrophic and the heterotrophic pathway, were differently affected by cycloheximide addition. At  $2.5\ \text{mg g}^{-1}$  cycloheximide, heterotrophic nitrification was completely blocked while autotrophic nitrification was still significant. If cycloheximide was sufficiently toxic to prevent any kind of nitrification at  $2.5\ \text{mg g}^{-1}$ , we would have not observed an active autotrophic nitrifiers



microflora, especially because autotrophic nitrifiers are generally one of the most sensitive components of soil microflora. This suggests that not only fungi might represent the main component of the heterotrophic nitrifiers but also might contribute to the process of autotrophic nitrification. This would imply the existence of an autotrophic nitrification pathway in fungi. Further support to this hypothesis comes from the experiment of section § 5.1, where it was found that  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  production stimulated by peptone addition was significantly reduced by both low cycloheximide concentrations and/or 0.1% (100Pa) acetylene, which clearly demonstrate the role of ammonia monooxygenase in the pathway of nitrification. No evidence exists for the presence of the ammonia-monooxygenase enzyme in fungi. However, the presence of an enzyme very similar to ammonia-monooxygenase has been demonstrated in the heterotrophic bacterium *Tsa. Pantotropha* (Robertson and Kuenen, 1988). Autotrophic activity is usually defined as the oxidation of  $\text{NH}_4^+$  (mineral pool), while heterotrophic nitrification is usually defined as the  $\text{NO}_2^-$  and/or  $\text{NO}_3^-$  production from the organic N pool. The possibility that heterotrophic microorganisms such as fungi might uptake low molecular N compounds and deaminate them intracellularly, or deaminate them in the space immediately adjacent to the cell membrane, and then direct the  $\text{NH}_4^+$  in the cell into a pathway similar to the one described for autotrophic bacteria (Wood, 1986) might explain the results obtained. This, of course, is a speculation and more evidence is needed.

Results, however, need to be interpreted cautiously as they present two main problems. The first problem is that cycloheximide has been rarely used to evaluate the origin of  $\text{N}_2\text{O}$  and, when tested to evaluate the contribution of fungi to nitrification, antibiotics have never produced, at low concentration, an inhibition of 100% (Schimel *et al.*, 1984; Killham, 1987; Landi *et al.*, 1993). A partial contribution of fungi to nitrification is probable and thus the problem of demonstrating the effectiveness of antibiotics in blocking only target microorganisms was not considered. In the presented experiments  $\text{N}_2\text{O}$  fluxes were completely blocked by 2 mg cycloheximide  $\text{g}^{-1}$ , implying a complete absence of bacterial  $\text{N}_2\text{O}$  production. Though the experimental procedure is the same as reported in many works in the literature (see §

2.6, § 4.2, § 5.1), this finding would be quite new and several objections might be posed against it. First, cycloheximide may interfere with the bacterial population of autotrophic nitrifiers. Data from chapters 4, 5 and 6 do not seem to support this idea as long as the concentration of cycloheximide is kept lower than  $2.5 \text{ mg g}^{-1}$ . A second objection is that peptone would not be a suitable substrate for autotrophic nitrifying bacteria to produce  $\text{N}_2\text{O}$ . This is not true, as when peptone was added most of the amino-groups were quickly deaminated, with consequent release of plenty of freely extractable  $\text{NH}_4^+$ , which might have been a suitable substrate for nitrifying bacteria. Third, it might be considered that the addition of peptone might have favoured the fungal activity, creating a competitive disadvantage for autotrophic bacteria. This might have been possible and is without any doubt one of the main limits of the experiment, though peptone has been frequently used in cultures and in soil (Van Gool and Schmidt, 1973; Van de Dijk and Troelstra, 1980; Schimel *et al.*, 1984; Papen *et al.*, 1989; Landi *et al.*, 1993) to stimulate heterotrophic activity versus autotrophic nitrification. However, even in this case the results still indicate a potential for fungal nitrification and  $\text{N}_2\text{O}$  production, which might be expressed in particular conditions.

The second problem is that acetylene as well as cycloheximide was found to inhibit  $\text{N}_2\text{O}$  production (§ 5.1). This has raised the possibility of a fungal pathway of  $\text{N}_2\text{O}$  production which might involve ammonia-monooxygenase. It is a common belief that fungi do not have the enzyme ammonia-monooxygenase, however in the literature relatively few hypotheses of fungal nitrification pathways have been put forward and no conclusive evidence has been produced. Inhibitors which are more commonly used to test such hypotheses must be used with caution, as it has been demonstrated that they can give misleading results (Kuenen and Robertson, 1987). For example, nitrapyrin, which is considered to inhibit only autotrophic nitrification, has been found to inhibit some fungal activity as well (Namir *et al.*, 1986). Also the use of pure cultures can be difficult. In soil extractions, a very low percentage of microorganisms is generally extracted (1-10%) (Nannipieri, pers. comm.). Even if by chance the right fungi were extracted, it might be possible that culture conditions,

which are so different from soil conditions, would not be optimal for the activity we want to observe. Moreover, an activity in soil can be the result of the interactions of more than one species of organism, which cannot be demonstrated in studies with pure cultures (Kuenen and Robertson, 1987).

### **8.3 ECOLOGICAL SIGNIFICANCE OF DIFFERENT BIOLOGICAL PATHWAYS OF N<sub>2</sub>O PRODUCTION IN THE SOILS STUDIED**

To correlate data from laboratory experiments with field data is generally quite difficult and can only be an approximation. In this thesis laboratory work has been conducted on soil and not on pure cultures. This, from one point of view, makes it possible to test some hypotheses in controlled experiments without altering completely the original conditions in which microbial populations live in the soil. From another point of view, it makes it more difficult to produce microbiological evidence to support the results. Both the approaches have advantages and disadvantages and none of them can be considered appropriate for a study of microbial ecology which realistically reflects field situations. However, a few suggestions come from the results of laboratory experiments to explain, at least in part, field data on N<sub>2</sub>O fluxes.

Field results suggested that more than one mechanism was present in the soil, which was responsible for N<sub>2</sub>O emissions. One source was constantly low throughout the year and showed a certain seasonality. A second source was represented by different processes which were yielding higher N<sub>2</sub>O emissions associated with “hot spots” of microbial activity, characterised by the presence of fresh organic matter and high concentrations of NH<sub>4</sub><sup>+</sup>. This was observed, however, only for the woodland soil.

The contemporary presence of NH<sub>4</sub><sup>+</sup> and organic matter might create a favourable condition for N<sub>2</sub>O production by both autotrophic nitrifiers and heterotrophic nitrifiers-denitrifiers. In the former case, the presence of ammonium would provide both the substrate for autotrophic nitrifying activity and the substrate for N<sub>2</sub>O production, i.e. NO<sub>2</sub><sup>-</sup>, while the organic matter in the same microsite might lower the oxygen tension, due to heterotrophic respiration, so that NO<sub>2</sub><sup>-</sup> is used instead of O<sub>2</sub> as

an electron acceptor. Burns *et al.* (1997) have demonstrated with  $^{15}\text{N}$  experiments that the highest peaks of  $\text{N}_2\text{O}$  production were associated with  $\text{NO}_2^-$  reduction more than with the sole  $\text{NO}_3^-$  reduction or  $\text{NH}_4^+$  oxidation, that  $\text{N}_2\text{O}$  production increased with increasing soil water content and that for soil below field capacity the main contribution was derived from nitrification. Laboratory experiments described in this thesis showed that very high  $\text{N}_2\text{O}$  fluxes were induced in the woodland soil by  $\text{NaNO}_2$ , alone or with glucose and it was concluded that two main sources could have been responsible for those fluxes, autotrophic nitrifiers and heterotrophic nitrifiers. In both cases microorganisms were supposed to reduce  $\text{NO}_2^-$  with production of  $\text{N}_2\text{O}$ , using  $\text{NO}_2^-$  instead than  $\text{O}_2$  as an electron acceptor if and when the conditions were favourable (Poth and Focht, 1985; Robertson and Kuenen, 1988).

For heterotrophic nitrifiers-denitrifiers the addition of  $\text{NH}_4^+$  would provide the electron acceptor  $\text{NO}_2^-$  and the organic matter might at the same time lower the oxygen tension in the microsite and be a substrate for heterotrophic nitrifiers. These might use  $\text{NO}_2^-$  as an alternative electron acceptor to allow a faster rate of NADH reoxidation, produced during the respiration of the organic substrate when  $\text{O}_2$  concentration is low (Robertson *et al.*, 1988). A direct implication of denitrifiers has been excluded (see discussion of § 4.1).

In the woodland the mineral N, an in particular extractable  $\text{NH}_4^+\text{-N}$  was always extremely low. The measured low fluxes might have been due to nitrification of the small quantities of  $\text{NH}_4^+$  mineralized by the microbial biomass, or might derive from the direct oxidation of organic-N by heterotrophic nitrifiers. In both cases  $\text{N}_2\text{O}$  fluxes would not be influenced by one special factor but by the overall combination of environmental parameters which influence microbial activity in soil. This would explain both the exhibited seasonal trend of  $\text{N}_2\text{O}$  fluxes and the lack of correlation between  $\text{N}_2\text{O}$  fluxes and a particular environmental parameter.

As suggested by the experimental results the heterotrophic component of soil nitrifiers might be quite important in the woodland soil and fungi might represent its major component. Though in laboratory observations peptone-induced  $\text{N}_2\text{O}$  fluxes were

quite low, this does not necessarily mean low net rates of fungal nitrification throughout the year, as, especially in forest soils, fungal biomass may be exceptionally high (Baath *et al.*, 1980) and active during the overall year. It is possible that in situations where autotrophic nitrifiers do not have a competitive advantage, the contribution of heterotrophs to soil nitrification might be significant. The efficiency and the extent of microbial utilization of a substrate in soil depend on the substrate diffusion (Skopp *et al.*, 1990), on the enzymes and on the movement of soil microorganisms. Microorganisms which are able to explore pore and soil aggregate will be able to utilise substrates more easily, especially those which are less soluble. Fungal mycelia are able to grow quite rapidly along the walls of coarse pores or even across the pores to get to potential substrates (Griffin, 1972), even in the absence of a continuous water pathway which is the prerequisite for bacterial colonization of new soil aggregates (Adu and Oades, 1978). Moreover, the high surface area-to-volume ratio of fungal hyphae in filamentous fungi (Waid, 1960) may favour fungal competition for mineral N and low molecular weight N compounds in soil, by direct uptake and by a more extensive distribution of extracellular enzymes responsible for the breakdown of macromolecules (Adu and Oades, 1978; Burns, 1982).

Experiments conducted on the woodland soil at WFPS between 60% and 100% (chapter 7) showed that for increasing water contents the fungal contribution to  $N_2O$  production decreased, the contribution deriving from nitrification increased up to 90% WFPS and contribution from denitrification was maximum around 100% WFPS. In the field, during the year '94-'95, the WFPS measured in the woodland was on average around the 60% and never exceeded 80%. In the arable soil it was even lower. It could be expected that fungi might have been in a favourable environmental situation during most of the year to compete with soil bacteria for N sources. Where "hot spots" of microbial activity, associated with organic matter, would lower oxygen tensions, nitrifiers-denitrifiers (both autotrophic or heterotrophic) and denitrifiers bacteria might be at a greater advantage and become a more important source of  $N_2O$ . These organisms might be able to produce much higher rates of  $N_2O$ , as demonstrated



in § 4.2 and they could be responsible for the peaks observed in the woodland field and soil cores.

Experiments with soil cores have showed that extremely low  $\text{N}_2\text{O}$  fluxes measured in the field in the arable soil might be due to low contents of C and mineral N, rather than to a lower microbial biomass. Indeed, even though the arable soil microbial biomass was much lower than in the woodland, it exhibited respiration rates comparable to those in the woodland, indicating a very active microflora. However, laboratory experiment (§ 4.1) showed that the arable soil had a very active denitrifier population, probably adapted to predominantly aerobic conditions. The presence of an active population of aerobic denitrifiers in a soil such as the studied arable soil is not improbable. The ubiquity of denitrifying bacteria has been illustrated by a study of Gamble *et al.* (1977), where denitrifiers were found to occur in soils over a wide range of texture, temperature, pH and moisture levels. Robertson and Kuenen (1984) have predicted that aerobic denitrifiers would have a selective advantage over specialists (obligate anaerobes) when oxygen was either fluctuating or limiting. The fact that in the wheat field fluxes were always so low might be due to the lack of optimal condition for  $\text{N}_2\text{O}$  production, i.e. contemporary presence of fresh organic C and mineral N. In the arable soil the C content was very low during most of the year. In the two periods when the input of organic matter was higher, i.e. in early summer when plants were fully grown (root exudates and root turnover) and in late summer when plants were cut (dead roots in soil), the concentration of mineral N was extremely low. The arable soil did not show any potential for direct  $\text{N}_2\text{O}$  production from an organic substrate such as peptone and it was much less sensitive to the fungal inhibitor cycloheximide, suggesting a minor role of fungi in this environment by comparison with the woodland soil. Bacterial autotrophic nitrifiers might be responsible for the low and constant fluxes measured throughout the year.

The yield of  $\text{N}_2\text{O}$  and  $\text{N}_2$  did not account for all consumed mineral N. It is possible that significant quantities of NO could have been produced. NO is produced both by denitrification and as a sub-product of nitrification, together with  $\text{N}_2\text{O}$  (Firestone and



Davidson, 1989). It is generally reported that the ratio  $\text{NO}:\text{N}_2\text{O}$  is very high in aerobic soil (Skiba *et al.*, 1992), so that the contribution of  $\text{NO}$  to the overall N balance might need to be quantified to avoid underestimation of N gas fluxes, especially in light textured soils.

#### 8.4 CONCLUSIONS

The results presented showed that different forms of N had very different impacts on  $\text{N}_2\text{O}$  emission in the two light textured soils, suggesting the involvement of different microbial communities in the  $\text{N}_2\text{O}$  production in the two environments. The arable soil had a very low potential for  $\text{N}_2\text{O}$  emissions deriving from nitrifiable N, as compared with the  $\text{N}_2\text{O}$  which was produced when the soil was provided with nitrogenous oxides and a carbon source. A very active population of denitrifiers, probably adapted to predominantly aerobic conditions, seemed responsible for the fast rate of nitrate consumption and  $\text{N}_2\text{O}$  production measured in the laboratory. It appears, therefore, that light soils could contribute to significant  $\text{N}_2\text{O}$  fluxes from denitrification, if fertilized with nitrate in the presence of readily decomposable organic matter. These conditions, however, were not frequent in the arable soil which showed extremely low fluxes during the whole year, which were probably due to bacterial oxidation of the small quantity of  $\text{NH}_4^+$  released by mineralization.

The woodland soil showed a very low denitrification activity, but a much higher  $\text{N}_2\text{O}$  production via  $\text{NH}_4^+$  oxidation and reduction of  $\text{NO}_2^-$  by some process mediated by autotrophic or heterotrophic nitrifiers.  $\text{NH}_3$  deposition in light textured forest soils could then stimulate  $\text{N}_2\text{O}$  emissions if  $\text{NO}_2^-$  accumulates in microsites, and this process could be enhanced by the presence of ammonium (autotrophic  $\text{NO}_2^-$  reduction) and/or organic carbon (heterotrophic nitrification). Heterotrophic microorganisms could be an important component in such processes. In the woodland soil, fungi seemed to be involved in the  $\text{N}_2\text{O}$  production via an organic route. However, acetylene reduced those emissions and some reduction in the  $\text{N}_2\text{O}$  production via an inorganic route (ammonium oxidation) was induced by the addition of cycloheximide. Two possible

explanation are: a) fungi can nitrify by both an organic and an inorganic route and ammonia monooxygenase might be involved in the pathway of  $\text{N}_2\text{O}$  production; b) the methods used to demonstrate a fungal role in the  $\text{N}_2\text{O}$  production could interfere with bacterial autotrophic nitrification and  $\text{N}_2\text{O}$  production. The data presented in the thesis support to the first hypothesis, however they are not conclusive and more research is needed.

## 8.5 FURTHER WORK

The presented results have raised several questions which might be the basis for further studies.

- Experiments with antibiotics and  $^{15}\text{N}$  have provided some evidence that cycloheximide and streptomycin might represent a source of C and N for microbes as indeed mineralization and respiration rates were stimulated by the addition of antibiotics. It might be interesting to follow the dynamic of mineralization and nitrification in samples treated with antibiotic labelled with  $^{15}\text{N}$  and  $^{14}\text{C}$  with a sampling frequency of few hours.
- It might be also interesting to follow the microbial community dynamics when the soil is treated with antibiotics at different concentrations to understand how and how much the microbial community changes from the moment of antibiotic addition onwards. It would be particularly meaningful to compare such changes with changes of microbial activity over time in order to understand for how long and at what extent the physiological block techniques reflect a situation similar to the moment of sampling.
- In the present experiment only peptone was tested as an organic substrate to evaluate the potential for heterotrophic nitrification in the woodland soil. Further studies might include the use of a much wider range of organic substrates labelled with  $^{15}\text{N}$ . Enrichment and dilution techniques and the use of a GC coupled with a mass spectrometer would allow to follow the dynamic of mineralization, autotrophic and heterotrophic nitrification and to determine how much of the

added organic-N will be lost as  $\text{N}_2\text{O}$  or  $\text{N}_2$ . This would help to have a much wider picture on the potential of this soil for heterotrophic nitrification and heterotrophic  $\text{N}_2\text{O}$  production.

- A more accurate study with pure culture would also be required to investigate the possibility of inorganic pathways of fungal nitrification, the sensitivity of this process to low concentrations of  $\text{C}_2\text{H}_2$  and the existence of an enzyme similar to the ammonia-monooxygenase also in eukaryotic heterotrophic microorganisms.

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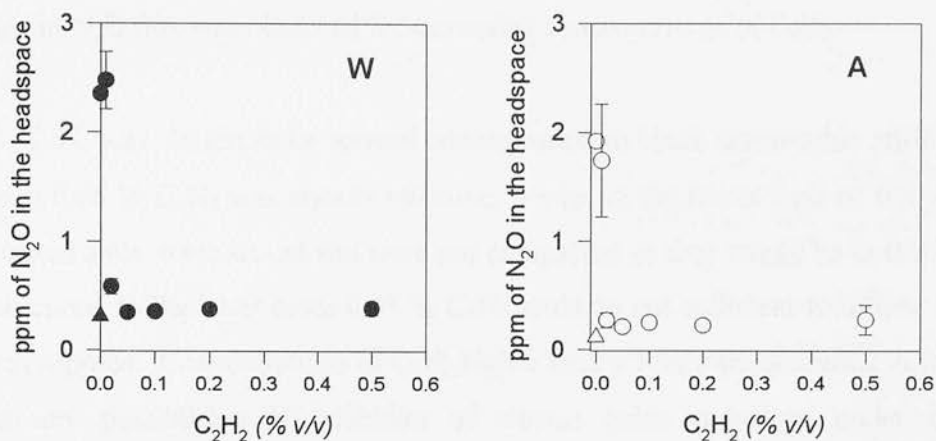


### **TEST FOR INHIBITION OF AUTOTROPHIC N<sub>2</sub>O PRODUCTION BY C<sub>2</sub>H<sub>2</sub> IN THE WOODLAND AND THE ARABLE SOIL**

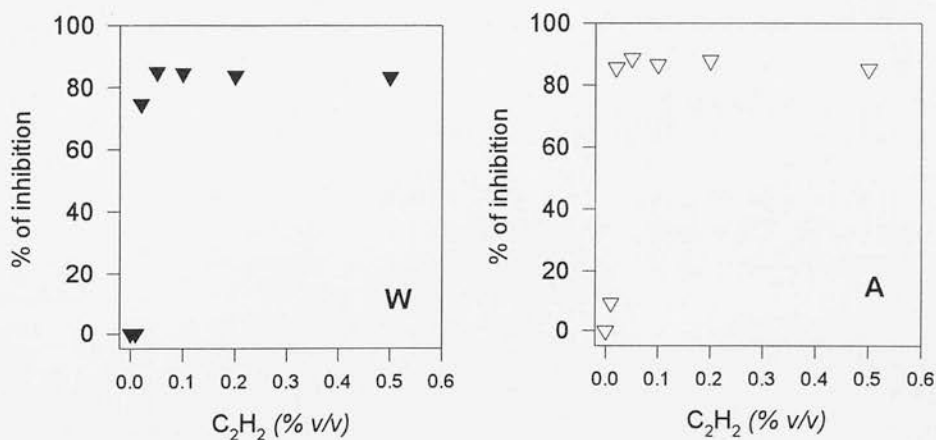
Before C<sub>2</sub>H<sub>2</sub> was used as an inhibitor of autotrophic N<sub>2</sub>O production, the woodland soil and the arable soil were tested to find out the optimal C<sub>2</sub>H<sub>2</sub> concentration which could enable to block ammonia-monooxygenase activity without inducing any secondary effect on denitrification, such as nitrous oxide reductase inhibition.

Soils were sampled from the top 30 cm, brought back to the laboratory and stored overnight at room temperature. The next day the soil was sieved ( $\varnothing$  2 mm) and adjusted at the 60% of WFPS. 70  $\mu\text{g}$  of N-NH<sub>4</sub>NO<sub>3</sub> g<sup>-1</sup> soil were added to each sample together with 750  $\mu\text{g}$  of glucose g<sup>-1</sup> soil (0.5 g talcum 100 g<sup>-1</sup> soil as carrier) to provide a potential substrate for both nitrification and denitrification. Samples of 50 g each, were incubated in 1 l air-tight jars. Immediately after closing the lids, 0, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 % (v/v) C<sub>2</sub>H<sub>2</sub> was added to the jars. Each treatment was done on 4 replicates. A control, (only soil on 4 replicates) was incubated together with the other samples. The same treatment was applied to both soils. Samples were incubated at 25°C for 24 hours. At the end of the incubation period, gas was sampled from each jar with air-thigh syringes and analysed by ECD chromatography to quantify N<sub>2</sub>O concentration in the sample (for all the routine methods see chapter 2).

Results are shown in Figure I.1. In both soils the addition of substrate induced significantly higher emissions than in the control. The addition of 0.01% C<sub>2</sub>H<sub>2</sub> was not effective in reducing the flush of N<sub>2</sub>O in both the woodland and the arable soil.



**Figure I.1** - ppmv of  $N_2O$  measured in the headspace of the jars of the treated samples (rounded symbol) and controls (triangles), from the woodland (W) and the arable (A) soils. Bars indicate one standard error.



**Figure I.2** - Inhibition % of  $N_2O$  emissions from the samples treated with  $NH_4NO_3$ , glucose and increasing concentrations of  $C_2H_2$  (0.0 - 0.5 % v/v).

Addition of 0.02% (v/v)  $C_2H_2$  induced a drastic reduction of  $N_2O$  emissions in both soils, though a significant further reduction was obtained in the woodland soil with 0.05%  $C_2H_2$ .

Inhibition in both soils was never more than about 85 % (Figure I.2). A plateau of inhibition (expressed as %) was already reached at 0.05 %  $C_2H_2$  and no significant change in  $N_2O$  flux was observed for increasing concentrations of  $C_2H_2$ .

0.1 %  $C_2H_2$  was chosen as an optimal concentration to block autotrophic nitrification. Though 0.05 %  $C_2H_2$  was equally effective, it was on the lower limit of the plateau. The tested soils were sieved and were not compacted as they would be in the field or in soil cores. In the latter cases 0.05 %  $C_2H_2$  could be not sufficient to diffuse into all the micropores. Concentrations of  $C_2H_2$  higher than 0.1 % were excluded in order to avoid any possibilities of inhibition of nitrous oxide reductase under optimal incubation conditions for denitrification activity.



## TABLES FROM CHAPTER 3 "FIELD EXPERIMENTS"

**Table II.1** - Values of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  measured in the treated and in the control plot from March 13<sup>th</sup> to April 4<sup>th</sup> 1995 in the woodland. Values in brackets are one standard error.

Soil depth (cm)	<i>TREATED</i>		<i>CONTROL</i>	
	$\text{NH}_4^+\text{-N}$ ( $\mu\text{g g soil}^{-1}$ )	$\text{NO}_3^-\text{-N}$ ( $\mu\text{g g soil}^{-1}$ )	$\text{NH}_4^+\text{-N}$ ( $\mu\text{g g soil}^{-1}$ )	$\text{NO}_3^-\text{-N}$ ( $\mu\text{g g soil}^{-1}$ )
<b>13-March</b>				
0 - 10	34.67 ( $\pm 11.49$ )	6.49 ( $\pm 0.33$ )	4.14 ( $\pm 0.75$ )	6.19 ( $\pm 0.09$ )
10 - 20	20.63 ( $\pm 15.51$ )	5.29 ( $\pm 1.88$ )	4.23 ( $\pm 0.65$ )	4.45 ( $\pm 0.11$ )
20 - 30	1.04 ( $\pm 0.07$ )	2.72 ( $\pm 0.37$ )	3.15 ( $\pm 1.26$ )	2.32 ( $\pm 0.08$ )
<b>14-March</b>				
0 - 10	61.06 ( $\pm 3.74$ )	7.98 ( $\pm 0.30$ )	2.04 ( $\pm 0.64$ )	5.06 ( $\pm 2.10$ )
10 - 20	18.02 ( $\pm 16.75$ )	6.13 ( $\pm 3.23$ )	1.14 ( $\pm 0.15$ )	5.90 ( $\pm 0.88$ )
20 - 30	1.17 ( $\pm 0.41$ )	3.73 ( $\pm 0.67$ )	1.32 ( $\pm 0.12$ )	5.33 ( $\pm 0.29$ )
<b>15-March</b>				
0 - 10	46.99 ( $\pm 8.79$ )	5.13 ( $\pm 0.54$ )	4.10 ( $\pm 0.40$ )	7.09 ( $\pm 0.09$ )
10 - 20	6.38 ( $\pm 3.97$ )	2.20 ( $\pm 0.34$ )	2.28 ( $\pm 0.24$ )	6.60 ( $\pm 0.36$ )
20 - 30	1.77 ( $\pm 0.30$ )	1.19 ( $\pm 0.02$ )	1.52 ( $\pm 0.01$ )	1.85 ( $\pm 0.08$ )
<b>16-March</b>				
0 - 10	71.59 ( $\pm 34.79$ )	12.13 ( $\pm 4.22$ )	1.87 ( $\pm 0.09$ )	3.40 ( $\pm 0.72$ )
10 - 20	4.54 ( $\pm 3.03$ )	4.03 ( $\pm 0.46$ )	2.06 ( $\pm 0.07$ )	3.83 ( $\pm 0.27$ )
20 - 30	1.66 ( $\pm 0.56$ )	2.47 ( $\pm 1.08$ )	1.13 ( $\pm 0.03$ )	1.74 ( $\pm 0.00$ )
<b>17-March</b>				
0 - 10	38.97 ( $\pm 18.25$ )	9.92 ( $\pm 1.51$ )	2.91 ( $\pm 0.09$ )	5.54 ( $\pm 0.93$ )
10 - 20	3.54 ( $\pm 1.42$ )	3.31 ( $\pm 0.95$ )	3.09 ( $\pm 0.19$ )	5.65 ( $\pm 0.06$ )
20 - 30	1.79 ( $\pm 0.04$ )	2.77 ( $\pm 0.58$ )	2.63 ( $\pm 0.12$ )	3.56 ( $\pm 0.07$ )
<b>22-March</b>				
0 - 10	30.23 ( $\pm 11.80$ )	20.38 ( $\pm 5.57$ )	2.15 ( $\pm 0.61$ )	6.98 ( $\pm 1.85$ )
10 - 20	9.70 ( $\pm 8.58$ )	11.72 ( $\pm 8.41$ )	2.00 ( $\pm 0.12$ )	4.77 ( $\pm 0.34$ )
20 - 30	1.07 ( $\pm 0.41$ )	2.30 ( $\pm 0.81$ )	1.63 ( $\pm 0.03$ )	3.51 ( $\pm 0.31$ )
<b>28-March</b>				
0 - 10	11.21 ( $\pm 4.02$ )	41.54 ( $\pm 8.79$ )	3.74 ( $\pm 0.22$ )	12.02 ( $\pm 0.26$ )
10 - 20	2.93 ( $\pm 0.60$ )	20.17 ( $\pm 11.51$ )	3.41 ( $\pm 1.57$ )	11.60 ( $\pm 4.00$ )
20 - 30	1.36 ( $\pm 0.10$ )	7.28 ( $\pm 1.99$ )	1.64 ( $\pm 0.04$ )	4.12 ( $\pm 0.02$ )
<b>31-March</b>				
0 - 10	6.34 ( $\pm 0.10$ )	7.10 ( $\pm 1.66$ )	3.72 ( $\pm 1.54$ )	14.51 ( $\pm 2.00$ )
10 - 20	1.76 ( $\pm 0.33$ )	4.82 ( $\pm 1.00$ )	3.14 ( $\pm 0.70$ )	12.09 ( $\pm 0.19$ )
20 - 30	0.80 ( $\pm 0.03$ )	3.45 ( $\pm 0.00$ )	1.72 ( $\pm 0.04$ )	13.23 ( $\pm 0.30$ )
<b>4-April</b>				
0 - 10	1.54 ( $\pm 0.12$ )	34.88 ( $\pm 3.46$ )	2.38 ( $\pm 0.49$ )	10.79 ( $\pm 0.50$ )
10 - 20	1.28 ( $\pm 0.19$ )	14.60 ( $\pm 6.59$ )	1.88 ( $\pm 0.14$ )	6.97 ( $\pm 0.25$ )
20 - 30	4.09 ( $\pm 3.62$ )	5.19 ( $\pm 2.70$ )	1.18 ( $\pm 0.05$ )	2.36 ( $\pm 0.02$ )

**Table II.2** - N<sub>2</sub>O emission rates from the woodland soil (loose samples, 0-20 cm depth) incubated with 0, 0.1% and 10 % C<sub>2</sub>H<sub>2</sub>. Values in brackets are one standard error.

	<i>0 % C<sub>2</sub>H<sub>2</sub></i>	<i>0.1 % C<sub>2</sub>H<sub>2</sub></i>	<i>10 % C<sub>2</sub>H<sub>2</sub></i>
<b>0 - 5 cm depth</b>	0.42 (± 0.20)	0.29 (± 0.08)	0.70 (± 0.10)
<b>5 - 10 cm “</b>	0.22 (± 0.03)	0.22 (± 0.05)	0.16 (± 0.03)
<b>10 - 15 cm “</b>	0.14 (± 0.02)	0.17 (± 0.01)	0.15 (± 0.05)
<b>15 - 20 cm “</b>	0.16 (± 0.00)	0.16 (± 0.01)	0.14 (± 0.07)

**Table II.3** - N<sub>2</sub>O emission rates from the arable soil (loose samples, 0-20 cm depth) incubated with 0, 0.1 and 10 % C<sub>2</sub>H<sub>2</sub>. Values in brackets are one standard error.

	<i>0 % C<sub>2</sub>H<sub>2</sub></i>	<i>0.1 % C<sub>2</sub>H<sub>2</sub></i>	<i>10 % C<sub>2</sub>H<sub>2</sub></i>
<b>0 - 5 cm depth</b>	0.07 (± 0.02)	0.11 (± 0.01)	0.07 (± 0.00)
<b>5 - 10 cm “</b>	0.08 (± 0.00)	0.07 (± 0.00)	0.08 (± 0.01)
<b>10 - 15 cm “</b>	0.07 (± 0.00)	0.07 (± 0.00)	0.07 (± 0.00)
<b>15 - 20 cm “</b>	0.07 (± 0.00)	0.07 (± 0.00)	0.09 (± 0.02)

### **THE CONTRIBUTION OF FUNGI TO NITROUS OXIDE RELEASE AND NITRIFICATION IN THE ARABLE SOIL**

Experiment in chapter 4, § 4.2, showed that when peptone was added to the arable soil very low  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  production was observed. It was concluded that heterotrophic activity was very low.

In this experiment the objective was to test the effect of cycloheximide on arable soil to estimate the fungal contribution to peptone-induced  $\text{N}_2\text{O}$  emissions and  $\text{NO}_3^-$  production and to confirm the minor role of fungi in those processes in this soil.

Soil was sampled and incubated as described in § 5.1.2, with the only difference that 0, 0.5, 1, 1.5, 2.5, and 3.5 mg of cycloheximide  $\text{g}^{-1}$  were added to the soil.

Results showed that the addition of peptone to the arable soil did not induce any significant emission of  $\text{N}_2\text{O}$  (Table III.1). Consequently, addition of cycloheximide and acetylene (0.1% v/v) did not have any appreciable effect on  $\text{N}_2\text{O}$  emissions (Table III.1).

These results differ from the results obtained in experiment of § 4.2 where peptone, added to the arable soil, induced a flux significantly higher than zero but still lower than in the woodland soil. However, from the bulk of the results, it was concluded that such flux could have been derived from the aerobic denitrification of the produced  $\text{NO}_3^-$ , which anyway was very low.

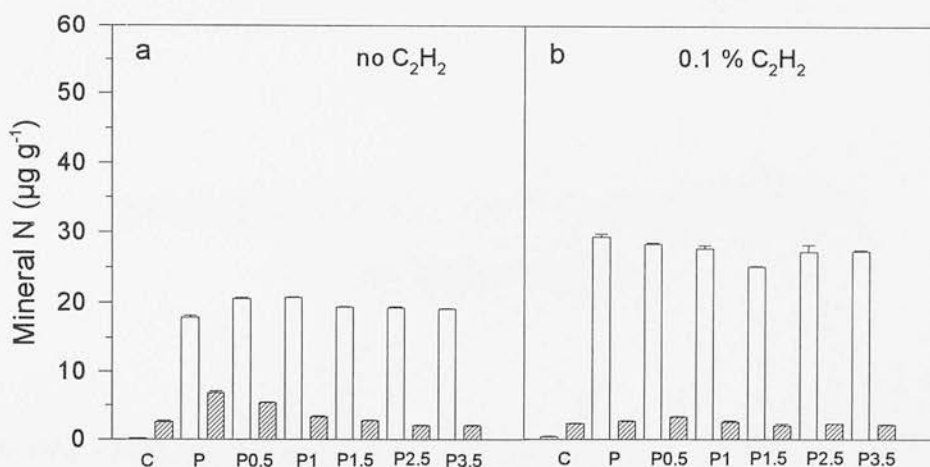
**Table III.1** - N<sub>2</sub>O emission rates from the arable soil untreated, treated with peptone or with peptone plus increasing concentration of cycloheximide, in presence of 0% or 0.1% C<sub>2</sub>H<sub>2</sub>. Values in brackets represent standard errors.

		Cycloheximide (mg g <sup>-1</sup> )	N <sub>2</sub> O-N (ng g <sup>-1</sup> h <sup>-1</sup> ) 0 % C <sub>2</sub> H <sub>2</sub>	N <sub>2</sub> O-N (ng g <sup>-1</sup> h <sup>-1</sup> ) 0.1 % C <sub>2</sub> H <sub>2</sub>
Untreated		0	-0.04 (± 0.00)	-0.05 (± 0.00)
Peptone (70 µg N g <sup>-1</sup> )		0	0.13 (± 0.06)	0.02 (± 0.07)
Peptone	"	0.5	0.01 (± 0.01)	-0.06 (± 0.04)
Peptone	"	1.0	-0.02 (± 0.00)	-0.02 (± 0.02)
Peptone	"	1.5	-0.05 (± 0.00)	0.03 (± 0.05)
Peptone	"	2.5	0.00 (± 0.02)	0.02 (± 0.02)
Peptone	"	3.5	0.03 (± 0.06)	-0.05 (± 0.12)

The net NO<sub>3</sub><sup>-</sup>-N production derived from peptone-N was about 4 µg g<sup>-1</sup>, only a tenth of that measured in the woodland soil (§ 4.1, § 4.2, § 5.1). 0.5 mg of cycloheximide g<sup>-1</sup> significantly reduced NO<sub>3</sub><sup>-</sup> production as compared with the treatment containing only peptone, and production was reduced to the control level (2 µg g<sup>-1</sup>) at 1.5 mg cycloheximide g<sup>-1</sup> (Figure III.1a). Addition of C<sub>2</sub>H<sub>2</sub> blocked NO<sub>3</sub><sup>-</sup> production at the control level in all treatments (Figure III.1b).

NH<sub>4</sub><sup>+</sup> accumulated in soil when peptone was added (18 µg of NH<sub>4</sub><sup>+</sup>-N g<sup>-1</sup>) and the concentration did not change significantly with cycloheximide addition. In the presence of C<sub>2</sub>H<sub>2</sub> the NH<sub>4</sub><sup>+</sup> concentration increased to 25 µg of NH<sub>4</sub><sup>+</sup>-N g<sup>-1</sup>. The quantity of NO<sub>3</sub><sup>-</sup> produced in the absence of acetylene accounted only for half of the NH<sub>4</sub><sup>+</sup> increase in presence of C<sub>2</sub>H<sub>2</sub>. Addition of cycloheximide did not induce further modifications.

The complete lack of N<sub>2</sub>O production, and the very small rate of NO<sub>3</sub><sup>-</sup> production, in the arable soil, after peptone addition, confirm the minor role for the fungal population in peptone degradation and N<sub>2</sub>O or NO<sub>3</sub><sup>-</sup> production in this soil.



**Figure III.1** - Available  $\text{NH}_4^+$ -N (open bars) and  $\text{NO}_3^-$ -N (shaded bars) measured, after 24 h, in the arable soil untreated (C), treated with peptone (P) or with peptone plus increasing concentration of cycloheximide expressed as mg of cycloheximide  $\text{g}^{-1}$  dry soil, in presence of (a) 0% or (b) 0.1%  $\text{C}_2\text{H}_2$ .

Generally in uncultivated soils, fungi are particularly abundant in the litter layer and in the uppermost few centimetres of soil, where there is plenty of organic matter, in the form of complex substrates, to decompose. However, the arable soil studied is a loamy sand, with a very low content of C, very low water content, no input of organic fertiliser and little or no aggregation. All these characteristics contribute to a much lower biomass as compared with the woodland soil ( 1:3 biomass ratio arable:woodland), and probably the conditions for fungal growth are much less favourable in the arable than in the woodland soil.

The very small contribution to  $\text{NO}_3^-$  production could derive from fungal activity as even 0.5 mg cycloheximide  $\text{g}^{-1}$  was effective in reducing the  $\text{NO}_3^-$  production. As  $\text{C}_2\text{H}_2$  reduced  $\text{NO}_3^-$  production as well, an inorganic pathway for fungal nitrification could be possible as discussed in chapter 5 and 6.

## MASS BALANCES FOR $^{15}\text{N}$ EXPERIMENTS OF CHAPTER 6

**Table IV.1** -  $\text{NH}_4^+$  mass balances for control (C) samples of experiment I. Cx:  $7.5 \text{ mg g}^{-1}$  cycloheximide; Str:  $3.5 \text{ mg g}^{-1}$  streptomycin sulphate;  $m$  and  $n$  are gross rates of mineralization and nitrification calculated with pool dilution equations (see chapter 6).

	C	C + Cx	C + Str	C + Cx + Str
$\text{NH}_4^+ (t=0)$	6.3	29.9	9.3	30.4
$+ m \times t^0$	8.7	19.2	11.9	13.7
$- n \times t^1$	7.2	0.2	8.7	1.7
calculated $\text{NH}_4^+$ pool	7.8	48.9	12.5	42.4
measured $\text{NH}_4^+$ pool	0.6	54.6	3.1	46.1
consumption	7.2	< 0	9.4	< 0

**Table IV.2** -  $\text{NO}_3^-$  mass balances for control samples of experiment I. For legends see Table IV.1.

	C	C + Cx	C + Str	C + Cx + Str
$\text{NO}_3^- (t=0)$	22.7	22.1	23.5	19.0
$+ n \times t^1$	7.2	0.2	8.7	1.7
calculated $\text{NO}_3^-$ pool	29.9	22.3	32.2	20.7
measured $\text{NO}_3^-$ pool	27.9	20.8	31.2	19.1
consumption	2.0	1.5	1.0	1.6



**Table IV.3** -  $\text{NH}_4^+$  mass balances for peptone (P) treated samples of experiment I. For legend see Table IV.1

	P	P + Cx	P + Str	P + Cx + Str
$\text{NH}_4^+$ (t=0)	20.2	38.6	24.2	38.0
+ $m \times t^0$	11.7	25.2	18.9	24.3
- $n \times t^t$	28.9	0.0	27.1	0.4
calculated $\text{NH}_4^+$ pool	3.0	63.8	16.0	61.9
measured $\text{NH}_4^+$ pool	1.0	60.3	8.9	70.8
consumption	2.0	3.5	7.1	< 0

**Table IV.4** -  $\text{NO}_3^-$  mass balances for peptone (P) treated samples of experiment I. For legend see Table IV.1.

	P	P + Cx	P + Str	P + Cx + Str
$\text{NO}_3^-$ (t=0)	27.2	22.9	28.6	21.4
+ $n \times t^t$	28.9	0.0	27.1	0.4
calculated $\text{NO}_3^-$ pool	56.1	22.9	55.7	21.8
measured $\text{NO}_3^-$ pool	60.2	22.7	57.2	21.4
consumption	< 0	0.2	< 0	0.4

**Table IV.5** -  $\text{NH}_4^+$  mass balances for control (C ) samples of experiment II. Cx: mg cycloheximide  $\text{g}^{-1}$  dry soil.

	C	C + 0.5 Cx	C + 1.5 Cx	C + 2.5 Cx
$\text{NH}_4^+$ (t=0)	3.6	6.0	8.9	15.2
+ $m \times t^0$	10.1*	10.1	17.1	15.1
- $n \times t^t$	6.6	8.2	9.1	7.3
calculated $\text{NH}_4^+$ pool	7.1	7.8	16.9	23.0
measured $\text{NH}_4^+$ pool	0.3	0.4	3.2	17.8
consumption	6.8	7.4	13.7	5.2

\* the value is not reliable

**Table IV.6** -  $\text{NO}_3^-$  mass balances for control (C ) samples of experiment II. Cx: mg cycloheximide  $\text{g}^{-1}$  dry soil.

	C	C + 0.5 Cx	C + 1.5 Cx	C + 2.5 Cx
$\text{NO}_3^-$ (t=0)	33.5	30.1	30.5	29.3
+ $n \times t^t$	6.6	8.2	9.1	7.3
calculated $\text{NO}_3^-$ pool	40.1	38.3	39.6	36.6
measured $\text{NO}_3^-$ pool	37.6	38.3	39.4	36.3
consumption	2.5	0.0	0.2	0.3

**Table IV.7** -  $\text{NH}_4^+$  mass balances for peptone (P) treated samples of experiment II. Cx: mg cycloheximide  $\text{g}^{-1}$  dry soil.

	P	P + 0.5 Cx	P + 1.5 Cx	P + 2.5 Cx
$\text{NH}_4^+$ (t=0)	80.4	83.0	81.2	82.9
+ $m \times t^0$	96.2	108.2	112.6	118.4
- $n \times t^1$	40.0	35.1	21.3	11.2
calculated $\text{NH}_4^+$ pool	136.6	156.1	172.5	190.1
measured $\text{NH}_4^+$ pool	106.0	123.8	146.9	168.9
consumption	30.6	32.3	25.6	21.2

Nitrate mass balances for peptone treated samples of experiment II have not been done because results were anomalous (see § 6.5.3).

### **TEST OF INHIBITING ACTION OF ANTIBIOTICS ON MICROBIAL GROWTH**

The aims of the experiment were: i) to test the effectiveness of cycloheximide and streptomycin in blocking the eukaryotic and prokaryotic growth, respectively; ii) to evaluate the effect of increasing concentrations of antibiotics on the growth of non-target microorganisms.

The technique used to test microbial growth was the “Dilution Pour Plate” technique. Soil was sampled from the top 20 cm in the woodland (Gullane), was sieved (2mm) and stored at 20°C. After few days 1 g of sieved soil was added to 100 cc of sterile water and microorganisms were extracted by sonication (2 min at 18,000 cycles/s frequency, i.e. tune 20  $\mu$ ). At the end of sonication soil looked quite mineral, with a few pieces of organic matter still floating in solution. From this mother solution 1 ml was taken and dissolved in 100 cc sterile water, and then once again, in order to have one solution diluted  $10^{-4}$  and one diluted  $10^{-6}$ , to test fungal and bacterial growth respectively. 1 ml from each of the two solutions was added to a sterile petri dish (on triplicate) and immediately after an agar media (8-10 cc) was added to the petri dish. Two different media were used: 1) potato-dexstrose agar (PDA) which was used for fungal growth and which was mixed with 1 ml of the  $10^{-4}$  solution, and 2) triptic agar (TSA), generally used for bacterial growth, which was mixed with 1 ml of  $10^{-6}$  solution. Before adding the agar media to the petra dish, containing 1 ml of soil extract, 1 ml of solution containing cycloheximide or streptomycin was added to the plates. A mother solution was prepared for each antibiotic (cycloheximide was diluted in 2/3 water and 1/3 ethanol). Both the solutions were sterilised by filtration using a sterile syringe provided with a sterile disposable filter (0,20  $\mu$  mesh), as autoclaving denaturates the antibiotics molecules. The mother solutions were used to prepare

other solutions of antibiotics (Table V.1) 10 times more concentrated than the desired final concentration, because of the dilution induced by the addition 10 ml of agar at the moment of the plating.

**Table V.1** - Final concentration of cycloheximide and streptomycin in the petra dishes before agar addition.

Cycloheximide mg ml <sup>-1</sup>	Streptomycin sulphate mg ml <sup>-1</sup>
5	5
15	15
25	25
35	35
75	-

Immediately after having added the agar medium the lids were closed and the plates were gently shaken. Then, plates were left to settle down and later incubated at 26 °C. Though a period between 7 and 14 days is usually required for a complete growth of all species of microorganisms, plates were incubated for 1 week only, because of a logistic problem, as the experiment was carried out in the Soil Science Department of Reading.

No fungal colony could be identified from the plates after 1 week of incubation, either if plated with PDA agar or with TSA agar. It is not clear why no fungal growth was observed, not even in the agar with the sole soil extract. A possible explanation could be that the pH of the agar medium was not appropriate. Material was standardly used for fungal growth and consequently pH of the medium was not measured before plating, which could have led to an error. Another possibility could be that the sonication was too strong and broke the fungal hyphae. Because of this failure it was not possible to evaluate the direct effect of antibiotics on fungi.

Bacteria and actinomycetes grew without problems in a week and, as expected, the growth was much higher on TSA agar than on PDA agar. In Table V.2 is reported the number of bacteria plus actinomycetes per gram of soil, counted using the dilution plate technique on TSA agar.

**Table V.2** - Bacteria plus actinomycetes counted from the plates of TSA agar after 1 week incubation. Values in brackets represent one standard deviation. In superscript different letters indicate significant differences ( $P < 0.05$ ).

Treatment for plating in TSA agar	Millions of prokaryotes g <sup>-1</sup> dry soil
Soil extract	40.8 <sup>a</sup> (± 14.4)
Soil extract + 0.5 mg cycloheximide ml <sup>-1</sup>	34.3 <sup>a</sup> (± 9.5)
Soil extract + 1.5 mg cycloheximide ml <sup>-1</sup>	26.8 <sup>a</sup> (± 3.7)
Soil extract + 2.5 mg cycloheximide ml <sup>-1</sup>	20.5 <sup>b</sup> (± 3.9)
Soil extract + 3.5 mg cycloheximide ml <sup>-1</sup>	16.8 <sup>c</sup> (± 7.3)
Soil extract + 7.5 mg cycloheximide ml <sup>-1</sup>	5.0 <sup>d</sup> (± 3.0)
Soil extract + 0.5 mg streptomycin ml <sup>-1</sup>	0 (± 0)
Soil extract + 1.5 mg streptomycin ml <sup>-1</sup>	0 (± 0)
Soil extract + 2.5 mg streptomycin ml <sup>-1</sup>	0 (± 0)
Soil extract + 3.5 mg streptomycin ml <sup>-1</sup>	0 (± 0)
Sterilized TSA agar (control)	0 (± 0)
TSA agar + 7.5 mg cycloheximide ml <sup>-1</sup>	0 (± 0)
TSA agar + 3.5 mg streptomycin ml <sup>-1</sup>	0 (± 0)

About 40 million prokaryotes per gram of soil were found in the woodland soil. When cycloheximide was added, the number of bacteria which were able to grow on plates decreased for increasing concentrations of cycloheximide. However, only for concentrations of cycloheximide higher than 1.5 mg ml<sup>-1</sup>, the number of colonies were reduced significantly. No significant difference was observed between colonies grown with 0, 0.5, and 1.5 mg cycloheximide g<sup>-1</sup>. All the tested concentrations of streptomycin blocked microbial growth; in all the plates treated with this antibiotic not a single colony was observed after one week incubation.



The results showed the efficacy of streptomycin in inhibiting prokaryotic protein synthesis and hence prokaryotic growth. The effect of streptomycin on bacterial growth in plates seemed stronger than what supposed from the results in previous experiment (§ 5.3, § 6.4). However, the concentration of streptomycin used in the plates was certainly higher than the concentration used in the soil incubation experiments. Moreover, in soil, microorganisms are more protected from the antibiotic action than they are in the plates once they have been extracted. What is interesting is that using cycloheximide and streptomycin in comparable concentrations produced as effect that in the low range of concentrations ( $< 2.5 \text{ mg g}^{-1}$ ) streptomycin significantly blocked bacterial growth while cycloheximide did not. This suggests that a massive biocidal or inhibitory effect of cycloheximide on bacterial growth is not observable at low concentrations, while at high concentrations cycloheximide becomes toxic.

It is important to underline that this experiment is not a direct and conclusive demonstration that low concentrations of cycloheximide do not have a detrimental effect on autotrophic nitrifying bacteria. It is well known that these bacteria are very slow to grow (several weeks) and that they require a special substrate as they are not good competitors (as compared with heterotrophic bacteria). Consequently, it is probable that not a single autotrophic nitrifier was present in the petri dishes. However, the results provide one more piece of evidence to support other data presented in the thesis, which suggest that cycloheximide needs to be at quite high concentration to start to have a clear destructive and biocidal action on the bacterial population and consequently the inhibition observed at low concentrations of cycloheximide could be the results of a selective block of protein synthesis on eukaryotic microorganisms. The fact that bacteria were extremely sensitive to streptomycin at a range of concentrations at which cycloheximide did not produce any significant inhibitory effect could further support the results of § 5.3.